Studies on the Occurrence of Antibiotic-Resistant Bacterial Strains from Agriculture-Associated Waters and their Proteomic and Genomic Characterizations

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I certify that this is a true and accurate version of the thesis approved by the examiners.

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Abstract

The serendipitous discovery of penicillin by Alexander Fleming in 1928 marked the glorious beginning of the modern antibiotic era with an innumerable number of lives being saved every single day by the use of antibiotics. Unfortunately, emergences of antibiotic resistance are taking us back to the pre-antibiotic era. Indiscriminate uses of antibiotics for therapeutic purposes and growth promotion in cattle are the major factors for the spread of antimicrobial drug resistance. Exposure of sub lethal levels of antibiotics to pathogenic microorganisms leads to the development of resistance, which then in due course spreads to other bacterial communities via horizontal and vertical gene transfer. Natural environment acts as a centre stage for such events and act as reservoir for antibiotic resistant bacteria. Anthropogenically polluted rivers can act as one such reservoir.

This thesis explores this possibility and describes the isolation characterization of multidrug resistant bacteria from the water samples of Dighty Burn. Five different strains of multidrug resistant bacteria viz. *Enterobacter* spp., *Burkholderia cepacia, Aeromonas hydrophila, Enterobacter agglomerans,* and *Pseudomonas stutzeri* that were resistant towards ampicillin, co-trimoxazole, novobiocin and tetracycline were successfully identified. Furthermore, the incidence of plasmid mediated and efflux pump mediated drug resistance has been proved in this work. Further studies involving proteomic and genomic analysis/comparison of environmental bacteria and their laboratory counterparts revealed the influence of the environment in conferring increased resistance and virulence in environmental strains as evidenced by marked variation in outer membrane proteins and virulence gene expression.

Thus the findings of the present study point out the role of our natural ecosystems, especially rivers, in spreading multidrug resistant pathogenic bacteria and warrants further study and proactive interventions to prevent the pollution of water bodies.

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Chapter 1. Introduction and Literature Review

Bacterial infections are associated with significant human suffering and mortality (NIH, 2011). They play a major role in human activity because of their ability to transfer quickly and effectively to susceptible individuals, causing morbid and fatal diseases. The human body is a reservoir for large numbers of microbes, especially bacteria. Most of these are non-pathogenic, but a few are opportunistic pathogens (Dethlefsen *et al.*, 2007). One well-known group of commensal bacteria in the gastrointestinal tract of mammals are the *Enterobacteriaceae* family. The organisms of this group help in the fermentation of undigested carbohydrates and production of vitamins in the gut (Wallace *et al.*, 2011). However, a few members of this family may cause significant disease in the form of food poisoning when conditions become suitable for them, e.g. lowered immunity etc. Alexander Fleming's discovery of penicillin in 1928 and the subsequent isolation and development of the first mass-produced antibiotic led to perhaps the greatest medical revolution of the 20th century. Many health problems and casualties during World War II were successfully treated with penicillin. The advent of antibiotics dramatically increased the average human life expectancy by eight years over the period between 1940 to 1972 (Smith *et al.*, 2013; Hollis & Ahmed 2013). However, antibiotics have been unknowingly used for thousands of years in certain traditional medicines as naturally occurring chemical substances with potent bactericidal properties (Oldfield & Feng, 2014). Fleming's discovery led to a golden age of antibiotic discoveries and widespread clinical usage of antibiotics became commonplace (Verbeken *et al.*, 2014).

Four years after Fleming's discovery of penicillin, the discovery of Prontosil further led to the development of sulphonamide drugs, another class of bactericidal drugs (Barr *et al.*, 1986). Sulphonamides and penicillin were first used in the 1930s and 1940s to treat bacterial infections,

yet evidence of antibiotic resistance was also shown by Fleming himself shortly after his discovery of penicillin (Yoneyama & Katsumata, 2006). Because of their ability to adapt quickly in response to external environment, bacteria soon developed resistance to the existing antibiotics as an evolutionary response to the challenges towards their growth and survival. For example, the *Shigella* spp. outbreak that occurred in Japan in 1953 led to the isolation of *Shigella dysenteriae*, a bacterial strain with multiple-drug resistance, which exhibits significant resistance against many drugs or antibiotics, such as sulphonamides, streptomycin, tetracycline and chloramphenicol (Watanabe, 1963) Resistance in bacteria can occur naturally, with recent research showing that bacteria exhibited antibiotic resistance long before Fleming's discovery (D'Costa *et al.*, 2011; Wardwell *et al.*, 2009) . In a study by D'Costa *et al.* (2011), bacteria isolated within permafrost dating back over 3,000 years ago were found to have markers of antibiotic resistance. Another study by Wardwell *et al.* (2009) found antibiotic resistant indigenous bacteria in a 2,000 year old peat bog habitat.

Even though some strains of bacteria naturally develop antibiotic resistance phenotypes, it is without question that bacteria over-exposed to antibiotics will eventually develop resistance towards the antibiotic as a natural survival mechanism (Davies & Davies, 2010). An example of this can be found in closely related families of bacteria, such as *Pseudomonas* spp. (Hibbing *et al.,* 2010). Substantial evidence suggests that irresponsible antibiotic administration in clinical institutions has been a major reason for the exponential growth of antibiotic resistant bacteria (Knobler *et al.,* 2003). In 2007, there were 400,000 reported infections and 25,000 reported deaths in Europe due to antibiotic resistant bacteria (Gillings, 2013). This has resulted in an estimated cumulative 2.5 million extra days in hospital and a societal cost of €1.5 billion annually

(Gilbert, n.d.). Therefore, drug resistant bacteria have become a significant medical and economic concern throughout the world.

1.1 Mechanisms of antibiotic resistance

Antibiotic resistance is often described as a simple evolutionary response to environmental pressures, when a bacterial colony is exposed to drugs, the cells that develop defences to these antibiotics will survive and multiply (Davies & Davies 2010). Antibiotic resistance in bacteria is a survival mechanism against adverse environmental conditions and can be either inherent as a natural feature of the species or can be acquired through a favourable mutation in the DNA or through exchange of genetic material coding for resistance to an antibiotic (Barr *et al.,* 1986). The success of any therapeutic agent, such as antibiotics, is compromised by the potential development of tolerance or resistance to the antibiotic in use. Resistance to antibiotics is a natural trait that occurs in some microbes, as in the case of Gram-negative bacteria in which the extra outer membrane acts as an additional barrier that reduces permeability for the entry of antibiotics (Ahmed *et al.,* 2010).

Antibiotics that have been developed to slow down and inhibit–bacterial growth–act in a number of ways, including the inhibition of cell wall synthesis (β-lactam antibiotics) (Cho *et al.*, 2014), inhibition of protein synthesis (streptomycin) (Luzzatto *et al.*, 1969) and the prevention of the replication of genetic material (ciprofloxacin) (Fournier *et al.*, 2000). In turn, *et al.,* several strains of bacteria have developed defence mechanisms tailored to counteract the functional mechanisms of these antibiotics, making these drugs inactive or ineffective in carrying out their functions (Davies & Davies, 2010). For example, changes in the number and character of porin channels that carry vital nutrients in and out of bacteria can lead to resistance to kanamycin (Hibbing *et al.,* 2010).

Inactivation of antibiotics by enzymatic action is another common way by which bacteria develop resistance to antibiotics (Davies, 1994). Some bacteria have enzymes that can modify the structures of antibiotics in ways that prevent its action against the microbe (Wright, 2005). Aminoglycosides, such as kanamycin and tobramycin, are inactivated by modifications that reduce the net positive charges on polycationic antibiotics (Davies & Wright, 1997), These processes involve phosphorylation catalysed by aminoglycoside phosphoryltransferase (APH), acetylation by aminoglycoside acetyltransferase (AAC), or adenylation by aminoglycoside adenyltransferase (Davies & Wright, 1997; Wright *et al.*, 1998; Magnet & Blanchard, 2005; Morar & Wright, 2010). On the other hand, penicillins, cephalosporins, and carbapenems, such as imipenem, are inactivated by enzymatic hydrolysis by β-lactamases which usually occurs in the bacterial periplasm (Li & Nikaido, 2009). The β-lactamase enzymes such as penicillinases degrade the penicillin drug by cleaving its β-lactam ring (D'Costa *et al.,* 2011). Genes coding for these inactivating enzymes can easily produce resistance as additional components on plasmids.

Some bacterial enzymes induce a physiological or structural change that alters the target site of the drug so that it is no longer bound by the antibiotic. These changes can come about through the selection for random point mutations during normal bacterial reproduction (Tenover, 2006). These mutations typically occur at a frequency of approximately 10^{-8} to 10^{-9}, or in other words, the common enteric bacteria develop resistance at the rate of one in a hundred thousand to one in a billion (Maiden, 1998).

1.2 Mutation and natural selection for antibiotic resistance

Mutation is a natural phenomenon that results in permanent changes in the genetic material brought about by alterations in the nucleotide sequences that make up the bacterial chromosome

or plasmid DNA. Some mutations remain unrepaired as they are not DNA damage *per se*, even though they can result from damaged DNA, but changes in the sequence of the nucleotide bases are replicated as the cell divides (Denver *et al.,* 2005).

In the presence of sufficient nutrients and in adequate environmental conditions bacteria continually replicate through binary fission, with each mother cell dividing into two genetically and phenotypically identical daughter cells (Holmes & Jobling, 1996). Therefore, each replication cycle results in a new generation of bacteria with the same DNA sequence as the original mother cell. When bacteria replicate in the presence of antibiotics (both anthropogenic and natural), mutations that naturally occur during the replication cycle may be selected for, leading to a new generation of bacteria that carry a slightly different genotype producing an antibiotic-resistant phenotype (Davies & Davies, 2010). These mutations are passed on to subsequent generations as each cell divides. This process of evolution in response to natural selection may result in mutated strains of a bacterial species that are more virulent or resistant to antibiotics than their non-mutant counterparts (Martínez & Baquero, 2002). However, not all mutations are favourable to the organism and even though the change is not detrimental their continued survival is very much dependent on the Darwinian theory of natural selection. Bacterial populations are in constant competition for food and habitat and those with the most favourable mutation to survive in antibiotic-laced environments have a reproductive edge over other species (Hibbing *et al.*, 2010). Mutation at the frequency of one in a billion would take a very long time to grant drug resistance in response to natural selection, but the rapid rate of bacterial replication, coupled with the enormous numbers of clones within a population overcomes the low mutation frequency and in a very short time results in antibiotic resistance within a population of bacteria (Levy, 2002). Rapid proliferation of bacteria aids the development

of resistance in mutant strains that eventually become dominant in an antibiotic-enriched environment. The next hurdle for a newly emergent antibiotic-resistant strain is to proliferate, as it is still under the risk of being destroyed by sudden changes in the environment where pure strains with the same genetic make-up tend to be more susceptible to antibiotic.

Recombination in bacteria (Schwesinger, 1977) also facilitates vertical gene transfer (VGT) with the added advantage of bringing in more genetic variation into the genome. However, widespread antibiotic resistance is not driven by vertical gene transfer alone, but by the acquisition of antibiotic resistant genes from other bacteria already having this property. This occurs by another phenomenon called Horizontal Gene Transfer (HGT).

HGT is possible because most of these antibiotic resistance mechanisms acquired through spontaneous mutations are encoded on extra-chromosomal DNA called plasmids that can be transmitted across bacteria of not only the same or closely related species, but to other distantly related species (Woodgate *et al.,* 1994).

1.3 Horizontal gene transfer and spread of antibiotic resistance

Horizontal gene transfer (HGT) is common in microorganisms, including bacteria, and results in the exchange of genetic material between several species, some of them distantly related (Nikolaidis *et al.,* 2014). The acquisition of a wide range of genetic adaptations favouring survival, such as antibiotic resistance, can be acquired more rapidly as a result of direct gene transfer compared to incremental inheritance of genetic mutations, which is also referred to as lateral gene transfer (LGT). This phenomenon is proving to be a challenge in the fight against antibiotic resistance in pathogenic bacteria. Multi-drug resistance, as well as increased virulence

in some of the most common pathogenic bacterial strains, has resulted from LGT of antibiotic resistance and exotoxin-producing genes (Koch, 2014).

The increasing virulence of drug resistant bacteria can be partly attributed to mutations as in the case of extended-spectrum β-lactamase-producing (ESBL) in the famous strain of *Escherichia coli* (ST131) that is causing an epidemic of extra-intestinal pathogenesis all over the world (Price *et al.*, 2013). However, the Shiga toxin-producing capability acquired by *E. coli* is a classic example of HGT from the donor *Shigella* spp. bacteria (Ogura *et al.*, 2015). Normally, a commensal bacterial species, such as enterohemorrhagic *E. coli* O157:H7 (EHEC) develops a virulent phenotype by adopting genetic information from *Shigella* sp. that produces the toxin (Price *et al.*, 2013).

HGT takes place through various mechanisms, such as transformation, conjugation and transduction. Other gene transfer and insertion agents, such as plasmids, bacteriophages, integrons and transposons may also play a role in the widespread occurrence of antibiotic resistance through HGT (Tennstedt *et al.*, 2005) Another phenomenon that favours the drug resistance acquisition across bacterial species is the clustering of these genes to form Complex Resistance gene Loci (CRL), making its transfer as well as dissemination within the microbe much easier, efficient and can explain the formation of bacterial strains with multi-drug resistance (Szczepanowski *et al.*, 2005).

1.4 Transformation

Transformation is a mechanism naturally found in many bacteria by which they directly take in genetic material from the surroundings and incorporate it into their own genome, resulting in the alteration of the genetic makeup of the organism (Cohen *et al.*, 1972; Griffith, 1928).

Transformation requires only one active player as it involves the acquisition of genetic materials from the environment where this material may be present in abundance, through cell death and disintegration that takes place especially when an organism has been in a competitive mode for survival. However, for transformation to occur, the bacterium requires high energy expenditure and should also be in a specific state of transformation competence (Leisner *et al.,* 2009). Limitation of nutrients and high population density are some of the factors that lead to the state of competence required. For example in *Bacillus subtilis*, scarcity of amino acids at the end of exponential growth brings about the competence for transformation. The intake of genetic material is likely to occur only in a specific set of conditions, which includes the presence of compatible material. Because of the restrictive nature of transformation, this method is not considered a great driving force in widespread antibiotic resistance. In several species of *Streptococcus*, transformation takes place in high-density conditions that result in the formation of bacterial biofilms that act as a reservoir of compatible genetic material (Li *et al.,* 2001).

1.5 Conjugation

Following the experimental verification of transformation as a horizontal gene transfer mechanism, in 1946 Joshua Lederberg and Edward Tatum discovered that a donor bacterium can transfer its genetic material to another donor bacterium after establishing a physical contact through a bridge or pilus (Amábile-Cuevas & Chicurel, 1993; Lederberg & Tatum, 1946). The genetic material is in the form of a plasmid, which is capable of independent replication. This horizontal gene transfer is beneficial to the recipient bacterium as it facilitates the transfer of an antibiotic resistance phenotype from the donor to the recipient cell, which enhances the recipient's ability to withstand the selective pressures from the environment. The bacterium

possessing this copy becomes capable of transferring the donated genetic material to another recipient. Conjugation is a highly effective horizontal gene transfer mechanism with the potential to drive large scale development of drug resistance across bacterial species, because genetic transfer can take place between distantly related species. Since the process can occur multiple times and between multiple donor-recipient pairs, conjugation has the potential to result in bacterial strains with multiple drug resistance (MDR). Conjugation employs a mechanism that detects whether the recipient bacterium already contains genetic material of the same type. This ensures that only truly beneficial genetic codes are exchanged (Andam & Hanage, 2014). Certain essential coupling proteins, such as plasmid R388 TrwB, assist the transfer of DNA across the cell membranes for successful bacterial conjugation (Gomis-Rüth *et al.,* 2002). Plasmids are one of the most important agents of HGT as they can be exchanged between distantly related bacterial species, as in the case of IncP-1 and PromA group plasmids that drive HGT in soil bacteria (Zhang *et al.,* 2015).

1.6 Transduction

Transduction is a method of horizontal gene transfer which does not require direct contact between the donor and recipient bacteria, but involves a vector, most often viruses, capable of infecting bacteria also known as "bacteriophages" (Yasbin & Young, 1974; Zinder & Lederberg, 1952). These phages use the hosts' mechanism of genetic replication to make copies of the viral DNA. The virus containing the bacterial gene that codes for antibiotic resistance infects a new bacterial cell and introduces this genetic material (containing marker for antibiotic resistance) into the recipient. Occasionally, bacterial DNA gets packaged in a viral capsid and when these virions containing bacterial genes infect a new bacterium, the bacterial DNA recombines with the

genetic material of the new host. This is a form of generalized transduction that happens accidentally, but another process called headful packaging by a bacteriophage (Coren *et al.,* 1995) also leads to generalized transduction. The virus fills the nucleocapsid with both viral and bacterial DNA, but on infecting another bacterium, the bacterial DNA may form plasmids within the recipient or recombine with its genetic material. Specialized transduction may also occur in some cases when the bacteriophage attaches to the host chromosome to make copies, but when the prophage detaches from the chromosome it takes a part of the host chromosome lying close to the point of excision and carries it to the next bacterium it infects. This type of transduction is observed particularly in the virulent enterohemorrhagic *E. coli* O157 that has also acquired *Shiga* toxins 1 and 2 (Asadulghani *et al.,* 2009). Transduction occurs in several other pathogenic bacteria species such as *Staphylococcus, Salmonella, Pseudomonas*, as well as in other *Escherichia* strains. Bacteriophages that mediate transduction can be virulent or temperate. Transduction can take place across families too. However, in most cases, it usually takes place between closely related bacterial species because of the involvement of the bacteriophages that may be species specific to some extent, which makes it a limited pathway for the spread of antibiotic resistance (Asadulghani *et al.,* 2009).

Whatever may be the way of gene transfer, the development of antibiotic resistance occurs only when the gene expresses itself and produces a significant effect, which results in the loss of activity of the antibiotic.

1.7 Acquired antibiotic resistance in natural environments

Resistance genes are not only distributed randomly in bacterial population, but also clustered in multiple drug resistance strains. Anthropogenic activities play a significant role in the distribution

of antibiotic resistance. Drug resistant strains initially appeared in hospitals, where use of antibiotics is common and in large quantity (Davies & Davies 2010). However, the frequency of international travel, combined with worldwide lack of standards of antibiotic use, generation and illegal methods of dumping hospital waste and sewage, has accelerated the spread of resistance across the globe and in every environment (Allen *et al.*, 2013; Levy, 2002). Antibiotic resistant bacteria are abundant in clinical environments and in farms of food producing animals (Vieira *et al.*, 2011) as well as in the food we consume (McDermott *et al.*, 2002). However, they are no longer restricted to the immediate human environment. The rampant use of antibiotics has resulted in the accumulation of drug-resistant genomes in various natural environments, including water bodies of such as rivers (Amaya *et al.*, 2012), soil (D'Costa *et al.*, 2007) and wildlife including wild birds (Hasan *et al.*, 2012). In response to increasing antibiotic resistance, new drugs are being developed, but bacteria are proving to be rather adept in developing resistance to these drugs. Even bacteria that have had no direct exposure to the antibiotics exhibit resistant behaviour, probably because of HGT that helps them acquire resistant genes from other microbes that have already been exposed.

Animal husbandry is a major contributor to drug resistance in microbes, because of the indiscriminate use of antibiotics for medical and non-therapeutic purposes in the veterinary field (Allen *et al.*, 2013). Antibiotics are frequently used in animals as part of the process to manufacture meats. Agricultural practices, such as the use of manure containing both low concentrations of antibiotics and antibiotic-resistant bacteria exacerbate the situation further (Ghosh & LaPara, 2007). Soil and water bodies in proximity to large animal farms are contaminated by a variety of pathogenic bacteria that can freely exchange genetic material with other naturally occurring bacteria to acquire multiple drug resistance (Da Costa *et al.*, 2013).

Treated waste water released into flowing water bodies is another major cause of contamination of natural environments since they carry human and animal pathogens, including antibiotic-resistant strains of bacteria, as well as antibiotics in low concentrations (Berglund, 2015). A recent year-long study of the effluents released from waste water treatment plants in Spain (Ojer-Usoz *et al.,* 2014) found extended-spectrum β-lactamase-producing *Enterobacteriaceae* in 100% of the samples examined. This clearly shows that wastewater from various sources, including hospitals, needs to be disinfected far more meticulously than is being done, before releasing it to water bodies, because it has the potential of spreading antimicrobial resistance far and wide into natural environments. Use of farm derived animal waste as manure is a widespread agricultural and aquacultural practice that can create a high risk for disseminating antimicrobial resistant pathogens to the environment (Omojowo & Omojasola, 2013) and wildlife (Blanco *et al.*, 2009).

1.8 The role of livestock in antibiotic resistance and dissemination

Antibiotics are as essential in disease management in livestock as they are for treating bacterial infections in humans (Silbergeld *et al.*, 2008). Antibiotic use in domestic pets to treat a variety of bacterial infections ranging from diarrhoea, eye infections and otitis to serious respiratory diseases is common, but there are no fixed standards or uniformity in prescribing the drugs across countries (De Briyne *et al.,* 2013; Wegener 2003). Intensive use and misuse of antibiotics in the veterinary field has contributed to the occurrence of bacterial resistance, regardless of whether these microbes are pathogenic, commensals or wild strains. Over recent years, antibiotic use has increased significantly in animal farms where large scale, intensive farming of food producing livestock is the norm to meet the massive demand for meat and other animal products (Landers *et*

al., 2012). The presence of large host populations in a small area can result in bacterial epidemics that can have high economic impact, which necessitates the use of antibiotics at community level not only for the treatment of the infections at times of disease outbreaks, but also as a prophylactic measure against potentially dangerous diseases (Laxminarayan *et al.*, 2013).

A proportion of the therapeutic dose of an antibiotic given to an animal will remain un-metabolized and will then be excreted via urine and / or faeces. This will result in the environmental release of a high chemical load to the soil and water near large farms. The lower concentrations of the drug, while not preventing bacterial growth, will promote drug resistance in these microbes (Chee-Sanford *et al.*, 2009). There is evidence that β-lactam antibiotics, when used at lower concentrations, actually increase the rate of HGT in *Staphylococcus aureus* by conjugative transfer of plasmids (Barr *et al.,* 1986). These chemical compounds break down during the treatment of waste water effluents and reach areas where pathogenic bacteria occur, thereby mediating lateral transfer of drug resistance among these organisms. Studies have proven the role of zinc oxide in promoting the proliferation of antibiotic resistance through many mechanisms including co-selection (Bednorz *et al.,* 2013), but many countries continue to use zinc oxide as an additive in animal feeds because of its significant ability to control weaning diarrhoea (Holm, 1996). The European Food Safety Agency (EFSA) allows 150 to 250 ppm of this chemical in animal feed and it is the most frequently used additive in medicated livestock feeds (De Briyne *et al.*, 2014). While disease management through antibiotics is an inevitable part of food production from farm animals, economic forces have led to the use of antibiotics in the feed to promote rapid growth and faster weight gain. Antibiotic usage began with sub-therapeutic dosages, when studies revealed a marked improvement in growth in broiler chickens that were administered these drugs through feed (Elam *et al.* 1953; Jacobs *et al.* 1953). The effect

was attributed to the reduction of gut bacteria in response to the antibiotic administration through feed that also reduced nutrient loss resulting from the feeding of these organisms (Visek, 1978). The meat-producing animals were able to better utilize the nutrients in their food, resulting in higher growth rate (Gaskins *et al.*, 2002). The drugs were to be used at an optimum concentration that lies between the minimum effective concentration required for therapeutic effect and the minimum inhibitory concentrations (MIC) required for controlling the gut bacterial population. However, in practice, they were often used at much higher concentrations than the prescribed MIC. Prolonged use of low-dose antibiotics is found to enhance the development of drug resistance in bacterial populations exposed to them (Gilbert 2011). The use of antimicrobials as growth promoters is also known to prompt *Escherichia coli* strains to release *Shiga* toxins that increase cytotoxicity, as well as *Shiga*-toxin-2-converting bacteriophages (Köhler *et al.,* 2000) that can spread this capability to other bacteria through transduction. Consequently, many European countries have banned the use of antimicrobial agents in livestock feed (Maron *et al.,* 2013), but 100% compliance has not been implemented and the practice still continues in countries where no such ban exists.

Alternatives to in-feed antimicrobials for growth promotion are being explored, but a drawback is that the exact mode of action of these drugs as growth promoters is unknown. The reduction in gut flora and consequent reduction in nutrient absorption by these microbes may not be the actual reason of the growth promoting effect. It was found that even though animals consuming feed fortified with the antimicrobial agent tylosin initially had significantly reduced microbial counts in faecal matter compared to the control group, the difference evened out after a period (Barug *et al.,* 2006).

Another alarming trend in veterinary medicine is the unrestricted use of Critically Important Antibiotics (CIA) to treat animal infections (WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR), 2011). The ever increasing occurrence of antibiotic resistance in bacteria and the corresponding increase in virulence in many new strains of pathogens has prompted the WHO to set aside certain important classes of antibiotics exclusively for human use. These important classes of antibiotics include: cephalosporins of the 3rd and 4th generation, fluoroquinolones, glycopeptides, macrolides, oxalidinones, carbapenems, and lipopeptides as well as many of the newly developed antimicrobials (WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR), 2011). These are segregated for human use because they are essential for treating bacterial infections in humans such as meningitis and pneumonia. A voluntary survey of veterinary practitioners conducted on the use of CIA in veterinary practice across 25 countries in Europe, showed varying degrees of CIA usage in domestic pets and food animals (De Briyne *et al.,* 2014). CIAs were often prescribed for treating respiratory infections in cattle and urinary infections in cats. This has the potential to cause the development of drug resistant bacterial strains in animals that can easily spread to humans (De Briyne *et al.,* 2014).

In a cross-sectional survey on the antimicrobial prescription practices for horses among veterinarians in UK (Hughes *et al.,* 2013) it was found that veterinarians in first-opinion practices prescribed broad-spectrum antimicrobials, such as trimethoprim-sulphonamide combinations more often for equine infections, because of their ease of use, lower cost and apparent lack of side effects. However, high levels of resistance to trimethoprim-sulphonamides have been reported in equine pathogenic bacteria (Maddox *et al.,* 2012), particularly in enteric bacteria from faecal samples, which questions the efficacy of these antimicrobials in the treatment of equine

infections. Veterinary clinicians who followed the guidelines of Veterinary Medicines Directorate (VMD) as their main resource for drug prescription are found to be less likely to prescribe trimethoprim-sulphonamides (Ahmed *et al.,* 2010).

On the other hand, practitioners in referral hospitals are more likely to use 3rd and 4th generation cephalosporins and fluoroquinolones for treating equine infections. Although it is possible that this could be partly due to serious multi-drug resistant infections being brought to referral practices, probably after the failure of first-line treatments, the UK has not licensed fluoroquinolones for treating equine infections (Hughes *et al.,* 2013). The use of these critical antimicrobials by referral practices were relatively low at 3.2% of all antimicrobials prescribed, but it was still nearly 10 times more than other practices. Moreover, the fluoroquinolone enrofloxacin, now available as Baytril™ for oral administration, is becoming more popular in equine practice in UK (Argyle, 2013).

The frequent and often indiscriminate use of antibiotics in the absence of correct protocols and monitoring mechanisms promotes the evolution of drug resistant strains with greater frequency among livestock (Johnson & Russo, 2002). The most frequent epidemics are caused by enteric bacterial species, such as *E. coli*, which can impact human health by causing gastrointestinal tract infections (Johnson & Russo, 2002).

Clear guidelines for antibiotic use in veterinary practice can go a long way in reducing the quantity of antimicrobials used, as well as the use of third-line drugs as demonstrated in a Canadian veterinary teaching hospital (Weese, 2006). This also reduces the prevalence of bacteria with antimicrobial resistance as demonstrated in human facilities with such guidelines for optimum antibiotic usage (Bantar *et al.,* 2003).

Besides the risks posed by direct contamination of human populations with drug resistant bacteria, a more widespread contamination of the environment, particularly soil and water, occurs through the disposal of animal waste products, including carcasses of animals dying of infectious diseases and manure put to agricultural use (Heuer *et al.,* 2011). Large concentrations of drug resistant genomes coming together from various sources create the ideal competency conditions that trigger HGT through transformation and other means, facilitating the sharing of drug resistance to not only closely related bacterial species, but even to distantly related microbes in the soil and effluent that have not had any direct exposure to the antibiotics themselves (Heuer *et al.,* 2011).

The anthropogenic antibiotic resistance in bacteria thus drives the evolution of microbial communities with multi-drug resistance, altering or destroying the ecosystem dynamics and shaping different bacterial lifestyles, resulting in significant consequences to human health (EU, 2013; Pitout & Laupland, 2008).

Direct exposure to antibiotics is thought to be minimal in wildlife populations. However, wild animals can acquire a variety of drug-resistant bacteria through direct or indirect contact with domestic animals and humans (Radhouani *et al.*, 2014). Typical examples are pests such as mice, rats and raccoons that raid farms that house livestock for food and come into contact with animals and their waste products that could be harbouring a number of antibiotic-resistant pathogens (Harwood *et al.,* 2000). The most common of all is *E. coli.* The contamination of the water sources consequent to the mixing of the faeces of farm animals containing heavy loads of antibiotic resistant bacteria and ESBL, wild mammalian and avian populations are open to increasing exposure (Costa *et al.,* 2008). Due to their high adaptability to survival in various media, these primarily enteric bacteria are proving to be a novel form of environmental pollution

(Guenther *et al.,* 2011) as well as a biomarker for monitoring the distribution and effect of multi-drug resistant microbes on wildlife.

One peculiar example of antibiotic resistance from wildlife is the study by Wildlife biologists (Cristóbal-Azkarate *et al.*, 2014). One study conducted to assess antibiotic resistance in bacteria in wildlife showed that antibiotic resistance was isolated among the wild Howler monkeys and other wild animals living freely in the Mexican region of Veracruz under different degrees of human influence (Cristóbal-Azkarate *et al.,* 2014). These areas are isolated national parks and are rarely inhabited by humans. The prevalence of antibiotic resistant bacteria was not only seen in howler monkeys, but also in tapirs, a dwarf leopard, jaguarondis, jaguars and spider monkeys (Cristóbal-Azkarate *et al.,* 2014). These results point to the fact that antibiotic resistance is inherent in nature and can take place independent from human activities.

1.9 The role of river water as a reservoir for antibiotic resistance and dissemination

River water, being a direct source of drinking water for both wild and domesticated animal, can be a medium from which to acquire antibiotic resistance (Rinker *et al.*, 1988). The river water receives processed effluents from factories as well as water processed from wastewater treatment, which may contain a cocktail of different antibiotics and other chemicals. This makes the river water a reservoir of low concentrations of antibiotics (Biyela *et al.*, 2004). The constant presence of low-level antibiotic concentration will annihilate susceptible organisms present, while at the same time it selects for organisms resistant to the antibiotics present in the river water, thus making the river water an ideal breeding ground for antibiotic-resistant organisms. Water from rivers is also used for agriculture and grazing lands from which they might enter the bodies of

domestic animals like cattle and sheep and hence, the human population (Ash *et al.*, 2002). In a study in India it was found that the Ciprofloxacin concentration as high as 2.5mg/L were reported in river water downstream of a wastewater treatment plant. This river was found to receive water from 90 bulk antibiotic manufacturers. The antibiotics that were found in the river water included trimethoprim, ofloxacin, lomefloxacin, enoxacin and (Fick *et al.,* 2009). When these resistant bacteria are excreted via animal faeces, they spread this resistance into the environment. Hence, the establishment of regulatory measures in the processing of wastewater from industries is of utmost importance.

1.10 The role of hospitals in the dissemination of antibiotic resistance

Hospitals and clinics are no less the responsible for the spread of antibiotic resistance amongst bacteria. Patients infected with bacteria come into the hospital and contaminate the hospital environment with contagious organisms that have a high probability of being antibiotic resistant already (Flaherty & Weinstein, 1996). These bacteria contaminate and infect other people and spread the resistance to the normal opportunistic microbial flora of the body (Wood *et al.*, 1996). Establishing clear guidelines for antimicrobial prescription in human and veterinary clinics and hospitals, and discouraging the non-therapeutic use of antibiotics, may help reduce drug-resistant bacterial concentration in the immediate human environment. However, whether this will considerably reduce observed drug-resistance, that is already established in the genomes of drug-resistant bacteria, and subsequently, its further distribution into natural environments is debatable.

The question as to whether antibiotics arose before antibiotic resistance or vice versa is highly debatable. Alternatively, antibiotic resistance may have co-evolved at the same time, since bacteria that produce the antibiotic are resistant to the antibiotic they produce. Over time, this

trait is passed on to bacterial progenies which continue to collect mobile genetic elements that carry antibiotic resistance and eventually, they establish resistance to multiple antibiotics (Choffnes *et al.,* 2010). It is also difficult to say whether the exposure to antibiotics causes the resistance or it is a matter of chance and mutation that bacteria acquire resistance.

1.11 Multi-drug and super drug resistance among bacteria

Unrestricted antibiotic use results in the spread of multi drug resistance (MDR) among number of bacteria that cause human infections (Alanis, 2005). The best-known example of this is MDR *Mycobacterium tuberculosis* (TB), which is a deadly organism prevalent in urban as well as rural settings (Almeida *et al.*, 2003; Ferrazoli *et al.*, 2000; Streicher *et al.*, 2004; Zaman *et al.*, 2005). Hospitals are the source of many bacterial infections, such as those caused by *Pseudomonas aeruginosa, Burkholderia cepacia, Salmonella* spp. and *Staphylococcus aureus* (Lee & Greig, 2013; Lipowski *et al.*, 2008; Peleg & Hooper, 2010). The treatment options for these bacteria are restricted and may lead to longer duration of hospitalization and monitoring (WHO, 2015). The super-resistant strains have various characteristics, such as greater transmission rates, that have boosted their virulence capacity. TB bacteria are the models for human pathogen, since they exhibit antibiotic resistance at a high rate and are currently known to latently infect about 1/3 of the world's population (WHO, 2013). *M. tuberculosis* has become resistant to successively introduced antibiotics, such as rifampicin and isoniazid (Somoskovi *et al.*, 2001). Recently, in addition to the MDR strains of tuberculosis, XDR (i.e. extremely drug resistant) and totally drug resistant strains (Shah *et al.,* 2007) have been observed. The interesting feature of drug resistance in TB is that its origin is not anthropogenic, but it spontaneously evolved by random mutations (Davies & Davies 2010).

The overuse of antibiotics in the Gram-negative pathogens, such as *Escherichia coli, Klebsiella pneumoniae* and *Salmonella enterica*, has made resistance a common phenomenon in these bacteria (Hopkins *et al.*, 2005; Wiener *et al.*, 1999). This resistance has become so prevalent that none of the antibiotics of the β-lactam class are effective against these organisms (Shaikh *et al.*, 2015). In cases of hospital infections, *Pseudomonas aeruginosa, Acinetobacter baumannii* and *Staphylococcus aureus* are the prevalent resistant organisms (Barbe *et al.*, 2004; Enright *et al.*, 2002). *Pseudomonas aeruginosa* was earlier identified as an organism responsible for burn wound infections and has now become a serious threat as an opportunist pathogen (De Bentzmann & Plésiat, 2011). Methicillin-resistant *Staphylococcus aureus* (MRSA) is the leading causes of nosocomial infection (Maddox *et al.*, 2012). Acquiring infections from antibiotic resistant bacteria has made it even more difficult to treat patients in hospitals. These infections have a chance of spreading to the healthy individuals as well making them agents of bacterial transmission. More deaths are associated with MRSA than with methicillin-sensitive strains (Levy and Marshall 2004).

1.12 Purpose and scope of project

The primary aim of this project is to assess the significance of the natural environment in the development of antibiotic resistance in Gram-negative bacteria. The natural environment is the major habitat of the bacteria. Bacteria can gain resistance to antibiotics either by genetic mutations or by horizontal gene transfer. Multi drug resistance and presence of various virulence factors make bacteria potentially harmful and increases their invasiveness and transmissibility. Therefore we propose that multi-drug resistance and pathogenicity of bacteria go hand in hand. Gram-negative bacteria, owning their greater resilience power, can survive in harsh environments

and are therefore more likely to evolve drug resistance towards antibiotics. Consequently, this makes multi-drug resistant Gram-negative bacteria the most potent pathogens responsible for widespread epidemics.

In this project, we aim to identify the characteristics of antibiotic resistance among the *Enterobacteriaceae* family. Finding the mechanisms of resistance practised by bacteria is the first step to develop effective therapy against that strain. The capacity of bacteria to withstand varying concentrations of different antibiotics is assessed by measuring the minimum inhibitor concentration (MIC) of antibiotics. The resistant bacteria identified can be used for further studies to understand the mechanism of antibiotic resistance, which may in turn help in devising suitable strategies to overcome the resistance.

River water is assumed to be the largest reservoir of both bacteria and antibiotics making it a perfect breeding ground for the antibiotic resistant strains. Furthermore rivers are also the final destination of the downstream processed chemicals from industries and wastewater emanating from the drainage system after the treatment. River water is a major source of drinking water for a large number of animals and humans, so pathogenic bacteria that are present in such water can easily find its way to its host. In this project, we aim to isolate and identify new bacterial isolates from a river that display resistance and investigate the mechanism of drug resistance.

1.13 The aims and objectives of this research

- To investigate the contribution of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria.

- To isolate and identify Gram-negative bacteria from a natural water environment, that display multi-drug resistance.
- To determine the role of plasmids in multidrug resistance mechanism of the newly identified isolates.
- To identify the possible mechanisms of multidrug resistance by expressing the proteins coded by the genes in the putative resistance plasmid isolated from the newly identified isolates.

1.14 Hypothesis

- Hypothesis: That the environment serves as a reservoir of antibiotic resistant bacteria.
- H_1= the environment is source reservoir of antibiotic resistant bacteria.
- Null hypothesis: That the environment does not serve as a reservoir of the antibiotic resistant bacteria.
- H_0= the environment is not a reservoir of antibiotics resistant bacteria.

Chapter 2. Materials and Methods

2.1 Site for sample collection

Bacteria were collected from the Dighty Burn. Water samples were collected using the same procedure described for monitoring and providing special natural characteristics of watercourses as rivers (JNCC, 2014). The Dighty Burn is located in Dundee and Angus and flows from the Sidlaw Hills to enter the Tay estuary between Broughty Ferry and Monifieth. Samples were taken from this river at one site at the Strathmartine Road (**OS 54**; MR NO 378 353; **Figure 2.1**). The water sampling was performed on 13/11/2012 around 10.00am. The weather was partly cloudy with an average temperature of 7°C, 93% humidity, and 5 km/h wind speed. The site was chosen to provide a breadth of environments within the river based on location characteristics. The river is surrounded by many agricultural activities as well as many household activities that provide a rich environment for the growth of diverse microbial communities including multidrug-resistant bacterial species. The laboratory strains used in the study was procured from the national Collections of Industrial and Marine Bacteria (NCIMB), Aberdeen, UK.

2.2 Isolation and characterisation of bacteria in water samples collected from the Dighty Burn by membrane filtration technique

Two litres of water samples from the river was collected in two pre-sterilized bottles of 1000mL volume each, sealed and transported to the laboratory within 30 minutes from the time of collection for further processing. The Membrane Filtration Technique was used to analyse the microbial load of water sample collected and to isolate microorganisms for the study.

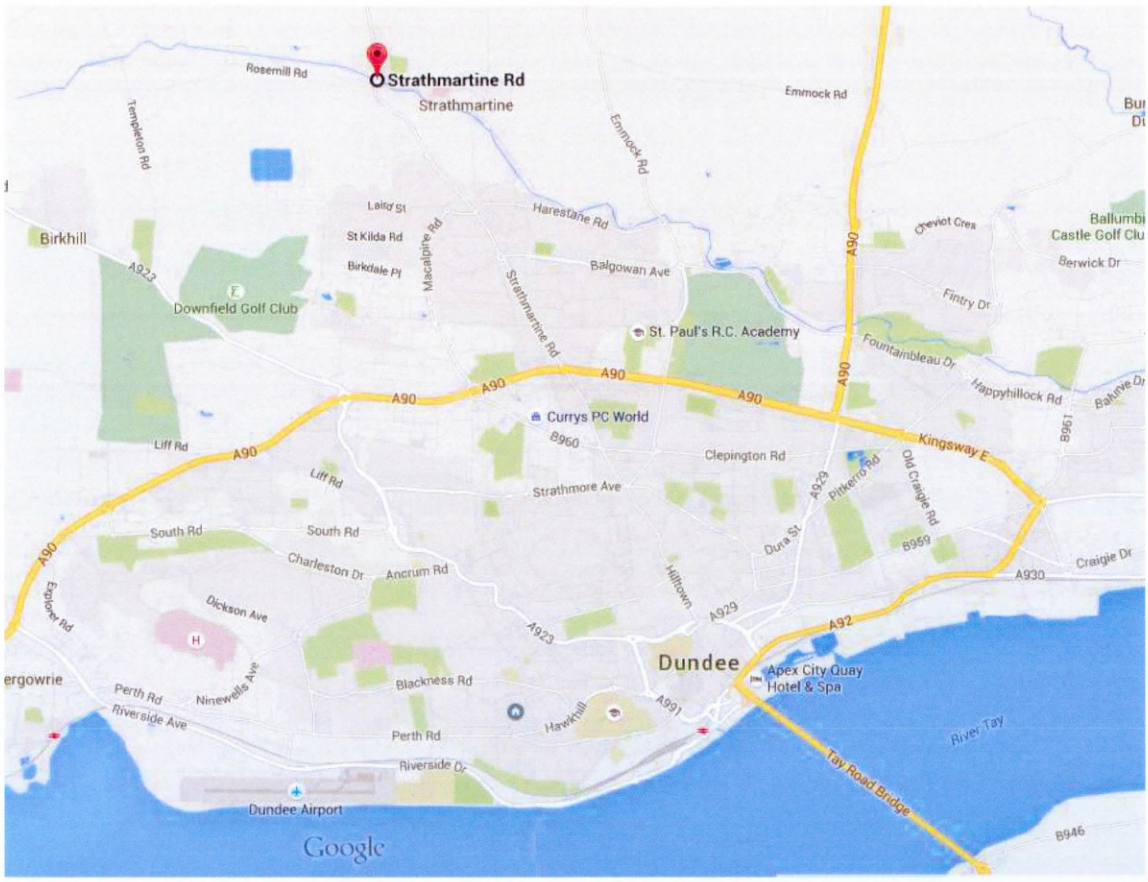

Figure 2.1: The Dighty Burn is located in Dundee and Angus and flows from the Sidlaw Hills to enter the Tay estuary between Broughty Ferry and Monifieth. (Water sampling location) Google maps (2015).

In the present study, bacteriological analysis was done using a membrane filtration technique following the procedure described in the Examination Guidance of Waters and Associated Materials (WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR), 2011). First, the membrane filtration apparatus was prepared by placing the sterile membrane filter on the filter base, grid side up and attached to the base so that the membrane filter is held between the sterile funnel and the base. This apparatus was then connected to the vacuum pump that facilitates the filtration process. Then, each of 100mL water sample was gently poured on the funnel and filtered through membrane the sterilized cellulose filters. During

filtration, the stop-cock was closed to avoid air being sucked through the membrane filter. After all the water samples were filtered, sterile water was gently poured into the sides to rinse the sides of the funnel and filter. After the water passed through the filter, the funnel was removed and filters were taken out with the use of sterile forceps and transferred to 47 mm diameter Petri plates previously prepared with Nutrient Agar (Oxoid, CM0003) or Malt Extract Agar (Oxoid, CM0059). Petri plates with Nutrient Agar (Oxoid, CM0003) were then incubated aerobically in incubator (Stuart orbital incubator.S150) or incubated anaerobically using an anaerobic atmosphere generation system Anaerogen jar (Oxoid LTD. AG25). Plates were incubated at either 25°C or 37°C for 48 hours. Petri plates with Malt Extract Agar (CM0059) were also incubated aerobically and anaerobically using the same incubations chambers at 25°C for 48 hours. After incubation the membrane filters were removed and the agar plates were examined for growth of colonies. **Figure 2.2** diagrammatically represents the membrane filter apparatus and the procedure followed.

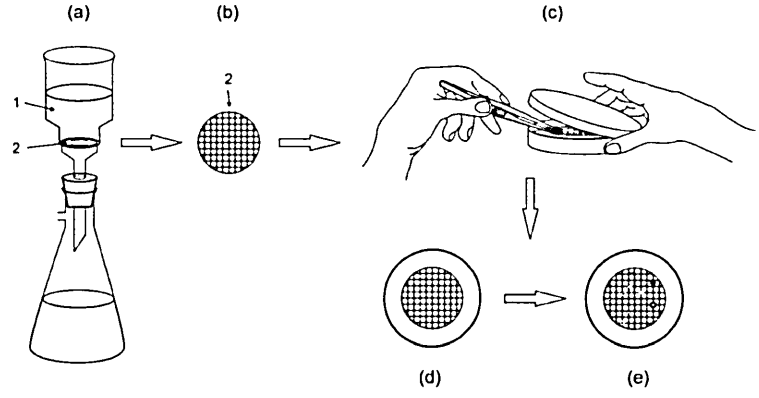

Figure 2.2: (a) Membrane filter apparatus, where (1) water sample and (2) filter. (b-c) Filter paper placed inside agar plate. (d-e) Bacterial colonies formed (http://elte.prompt.hu/sites/default/files/tananyagok/PracticalMicrobiology/ch05s03.html). (Accessed on October 27, 2015) (Attila Náfrádi, 2013) .

2.3 Viable plating techniques used for isolate bacteria from water samples

2.3.1 Sample preparation for isolation of bacteria and serial dilution

Ten mL of water samples were added aseptically to 0.9L of physiological saline (0.85% NaCl) or R2A medium and was thoroughly mixed. Two tablets of NaCl were dissolved in 1000mL of distilled water to prepare 0.85% of physiological saline. The preparation was incubated at 37°C for 24 hours when R2A medium was used. This was then serially diluted up to 12 tubes. The methodology followed is as given in **Figure 2.3**. The experiment was done in duplicates. This serially diluted suspension was used as the inoculums for various plating techniques. The isolated single colonies post incubation period was picked, and was purified on rich nutrient agar (NA) (Oxoid CM0003).

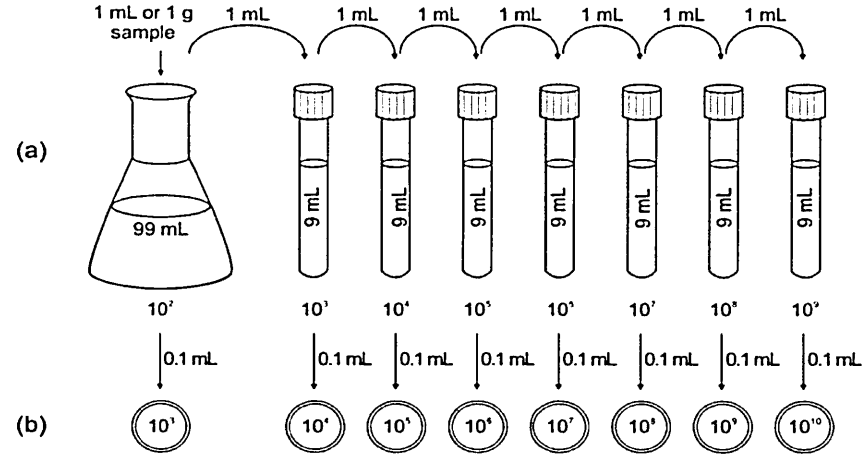

Figure 2.3: Serial dilution and pour plate method used to isolate bacteria from water sample. (a) The sample is diluted in sterile 85% saline, (b) 10-12 fold dilution series is prepared and (c) Appropriate amounts of these dilutions are plated onto suitable growth medium in the Petri plate if necessary. (http://elte.prompt.hu/sites/default/files/tananyagok/PracticalMicrobiology/ch05s03.html). (Accessed on October 27, 2015) (Attila Náfrádi, 2013).

2.4 Microbiological techniques used for isolating bacteria as pure cultures

2.4.1 Spread plate technique to isolate bacteria from serially diluted water samples

100 µl of the bacterial inocula from each of the serially diluted samples were placed on the centre of an agar plate and was spread over the surface of the agar, using a sterile glass rod. The glass rod was sterilized by dipping in alcohol (70% ethanol) and flaming it to burn off the alcohol. Individual microorganisms get separated from each and grow into individual colonies represent a clone of the pure culture. Best results were obtained when the bacterial inoculums were diluted properly. Spreading of undiluted samples may result in overcrowding and the formation of confluent growth. The diagrammatic representation of spread plate techniques is as shown in **Figure 2.4.**

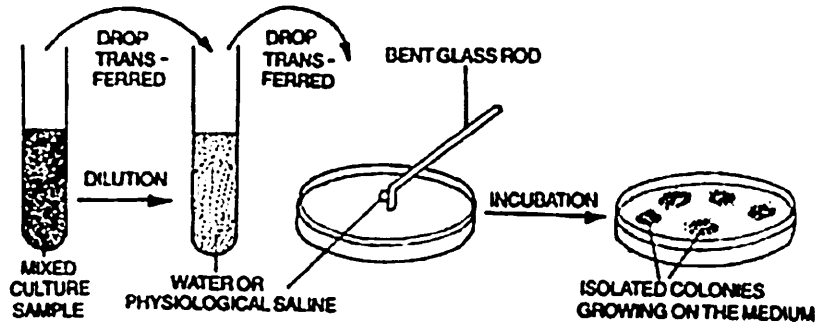

Figure 2.4: Spread plate method used to isolate and enumerate bacteria. (http://upendrats.blogspot.co.uk/2010/02/microbial-pure-culture.html). (Accessed on October 27, 2015) (Thapa, 2010).

2.4.2 Streak plate method to purify bacteria isolated through spread plate technique

This methodology was followed to ensure the purity of bacterial colonies isolated by spread plate. A loopful of inoculum is streaked across the surface of a sterile solidified agar plate that contains

a nutrient medium. The course of streaking on agar plate is as shown in **Figure 2.5**. The plates are then incubated under favourable conditions to permit the growth of the bacteria. A dilution gradient is established across the face of the plate as bacterial cells are deposited on the agar surface. Dilution gradient achieved by the method allow individual isolated colonies to develop in the plate region, which is farthest from starting region. Separate microscopic colonies that can visualize easily. Each of the well-isolated colony is assumed to arise from a single bacterium and therefore to represent a clone of the pure culture.

2.5 Stocking and maintenance of isolated and purified bacterial cultures

2.5.1 Temporary stocking of bacterial culture on nutrient agar slopes

The isolated organisms were maintained on rich nutrient agar (Oxoid CM0003) slopes in quadruplicate. Maintenance in multiple slopes helps in retrieving the culture in case of contamination. Slope number 1 was used for culturing overnight cultures for routine experiments. Slope number 2 was maintained as a backup slope to be used in case of accidental contamination of slope 1. Slope number 3 was used for further sub culturing purposes and slope number 4 was maintained as part of a stock culture collection. All the slopes inoculated with the bacteria were first incubated at 37°C for 24 hours. After incubation the slopes were transferred to a darkened cupboard maintained at room temperature. The slopes were renewed every month to maintain the viability and prevent the senescence of the cultures.

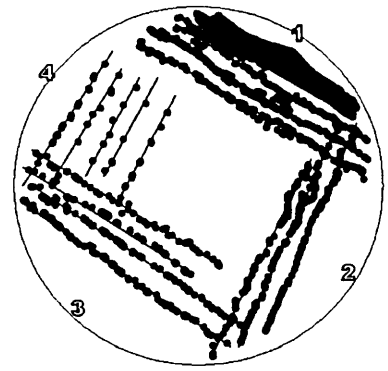

Figure 2.5: Streak plate method used to purify the isolated bacteria. (http://www.microbiol.org/resources/monographswhite-papers/streaking-for-single-colonies-an-essential-first-step-in-microbial-identification). (Accessed October 27, 2015) (Sutton, 2006).

2.5.2 Glycerol stocking of purified bacterial culture

The isolates were grown in R2A media. Supplemented with 1% glycerol (Reasoner & Geldreich, 1985) for 24 hours at 37°C in an orbital incubator (Gallenkamp, INA-SOS) at 220rpm After the incubation the broth was mixed with sterile glycerol to make 30% glycerol stock and kept at -80°C.

2.6 Identification and biochemical characterization of isolated bacterial cultures

2.6.1 Gram staining technique to differentiate bacteria into Gram-positive and Gram-negative classes

Slides were prepared by making a smear of the sub-culture on a clean, grease-free glass slide, followed by gentle heating to dry fix the film. Slides were initially stained with crystal violet dye for one minute and then flooded with Gram's iodine solution in order to form a complex between dye and iodine, technically referred to as a mordant, and left for another minute. The slides were then rapidly decolourized with acetone/alcohol until no blue stain was released from the slide.

Immediate rinsing under running water in order to remove the excess of acetone/ethanol then followed this. A secondary stain, safranin, was then added to the slides for 5 minutes and then slides are washed gently with a stream of water and blotted dry. The slides were examined under a microscope at 100X by using oil immersion lens. The following observations are expected:

i. Gram-positive Bacteria appear violet/purple.

ii. Gram-negative Bacteria appear red/pink

2.7 Biochemical characterization of bacterial isolates using ready-made strips: API 20E, AP1 20NE and API 50CH

API 20E, 20NE and 50CH (Biomerieux, France) was used for the biochemical identification of bacterial strains. API 20 NE strips were used to identify the non-Enterobacteriaceae, Gram-negative bacillus that is oxidase positive and non-fastidious in nature. API 20 E strips were used to identify the Gram-negative bacillus belonging to *Enterobacteriaceae* family, which are Oxidase negative and non-fastidious. API 50CH was used to identify Gram-positive *Bacillus* and related genera. API test strips consist of microtubes (cupules) containing dehydrated substrates. The tests are based on the principle of pH change and substrate utilization. On incubation, the metabolic changes are observed by spontaneous colour change in the media that can be interpreted visually. Assimilation tests are inoculated with a minimal medium (API AUX medium) and the bacteria grow if they are able to utilize the corresponding substrate: a positive result is indicated by growth. Test results are entered into an online database to determine the bacterial identity.

2.7.1 Inoculation of bacterial isolates on API 20E and API 20NE strips

A single well-isolated colony (2-3mm diameter) was picked up and inoculated into the saline tube (0.85% NaCl solution). This was then mixed well so that the suspension is homogenous and without clumps of floating.

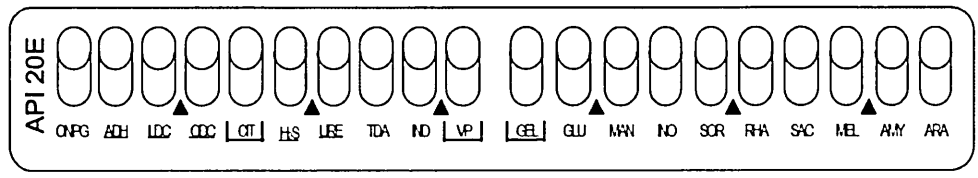

Figure 2.6: Schematic representation of the API 20E strip. Figure retrieved from instruction manual of API test strips (http://www.biomerieux-usa.com/clinical/api). (Accessed October 27, 2015) (Biomerieux, n.d.).

2.7.2 Procedure followed for inoculating API 20E strip

The strip was opened aseptically and labelled appropriately. Five mL of sterile distilled water was added into the tray to provide a moist atmosphere to prevent the drying of the strip. The strip was laid inside the tray. The strip was held at a slight angle up from the table top to inoculate the bacterial suspension into each well with the sterile pipette. The end of the pipette was gently introduced to the side of the cupule and inoculums were dispensed up to the neck cupules. After the inoculation the strips were incubated at 37°C for 18-24 hours. The schematic representation of the API 20E strip is as shown in **Figure 2.6**. The result of the test was read using the following **Table 2.1** and **Figure 2.7** that was provided with the strip.

The tests on the strip plus oxidase test result were used to determine the first seven digits of the profile number. The numerical profile thus obtained is then entered the APIweb™ (https://apiweb.biomerieux.com.accessed_11/03/2014) (Biomerieux, n.d.) to obtain the identity of the bacterial isolate.

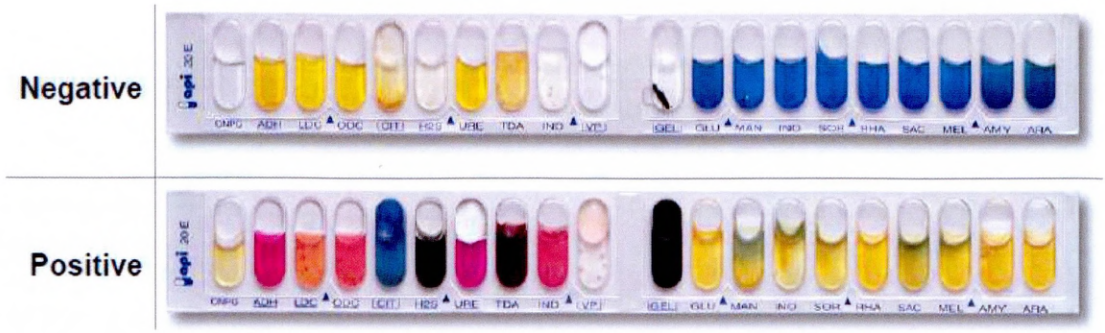

Figure 2.7: Positive and negative test results of API 20E strips. Figure retrieved from instruction manual of API test strips. (http://www.biomerieux-usa.com/clinical/api). (Accessed October 27, 2015) (Biomerieux, n.d.).

Table 2.1: Interpretation table provided with the API 20E strips for reading the results			
TEST	**REACTION**	**NEGATIVE**	**POSITIVE**
ONPG	β-galactosidase	Colourless	Yellow (maybe pale)
ADH	Arginine dihydrolase	Yellow	Orange or red
LDC	Lysine decarboxylase	Yellow	Orange or red
ODC	Ornithine decarboxylase	Yellow	Orange or red
CIT	Citrate utilisation	Light green	Blue-green or blue
H_2S	H_2S production	Colourless	Black
URE	Urea hydrolysis	Yellow	Pink
TDA	Tryptophan deamination	Yellow	Dark brown
IND	Indole production	Colourless reagent	Pink
VP	Acetoin production	Colourless	Pink or red
GEL	Gelatin hydrolysis	Colourless	Black diffuse pigment
GLU	Glucose fermentation	Blue	Yellow
MAN	Mannitol	Blue	Yellow
INO	Inositol	Blue	Yellow
SOR	Sorbitol	Blue	Yellow
RHA	Rhamnose	Blue	Yellow
SAC	Sucrose	Blue	Yellow
MEL	Melibiose	Blue	Yellow
AMY	Amygdalin	Blue	Yellow
ARA	Arabinose	Blue	Yellow
Oxidase	Cytochrome oxidase	Colourless	Purple

2.7.3 Procedure followed for inoculating API 20NE strip

The strip was opened aseptically and labelled appropriately. Five mL of sterile distilled water was added into the tray to provide a moist atmosphere, which prevent drying of the strip. The strip was laid inside the tray. The strip was held at a slight angle up from the table top to inoculate the bacterial suspension into each well with the sterile pipette. The end of the pipette was gently introduced to the side of the cupule and inoculums were dispensed up to the neck of capsules of

the NO_3 to PNPG microtubes. Tube and cupules of [GLU] to [PAC] where filled with bacterial suspension dispensed in AUX Medium. After the inoculation the strips were incubated at 37°C for 18-24 hours. The schematic representation of the API 20NE strip is as shown in **Figure 2.8** .The result of the test was read using the following **Table 2.2** and **Figure 2.9**.

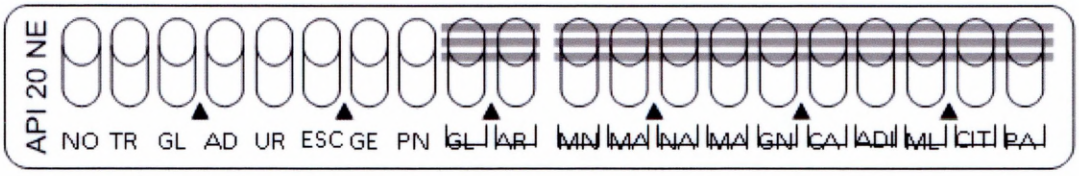

Figure 2.8: Schematic representation of the API20 NE strips. Figure retrieved from instruction manual of API test strips. (http://www.biomerieux-usa.com/clinical/api). (Accessed October 27, 2015) (Biomerieux, n.d.).

Figure 2.9: Positive and negative test results of API20 NE strips. Figure retrieved from instruction manual of API test strips. (http://www.biomerieux-usa.com/clinical/api). (Accessed on February 30, 2014) (Biomerieux, n.d.).

The tests on the strip were used to determine the first seven digits of the profile number. The numerical profile thus obtained is then entered the APIweb™ (Biomerieux, n.d.) to obtain the identity of the bacterial isolate.

Table 2.2: Interpretation table provided with the API20 NE strips for reading the results			
TEST	**REACTION**	**NEGATIVE**	**POSITIVE**
$NO_3 \rightarrow NO_2$ $NO_2 \rightarrow N_2$	Reduction of potassium nitrate	Colourless Red/pink	Red (NIT1+NIT2) Colourless (Zn)
TRP	Indole production from tryptophan	Yellow	Pink
GLU	Glucose fermentation	Blue/green	Yellow
ADH	Arginine hydrolysis	Yellow	Orange/pink/red
URE	Urea hydrolysis	Yellow	Orange/pink/red
ESC	Aesculin hydrolysis	Yellow	Grey/brown/black
GEL	Gelatin hydrolysis	No pigment diffusion	Diffusion of black pigment
PNPG	p-nitrophenyl-βD-galactopyranoside hydrolysis	Colourless	Yellow
GLU	Glucose assimilation	Transparent	Opaque
ARA	Arabinose assimilation	Transparent	Opaque
MNE	Mannose assimilation	Transparent	Opaque
MAN	Mannitol assimilation	Transparent	Opaque
NAG	N-acetyl-glucosamine assimilation	Transparent	Opaque
MAL	Maltose assimilation	Transparent	Opaque
GNT	Gluconate assimilation	Transparent	Opaque
CAP	Caprate assimilation	Transparent	Opaque
ADI	Adipate assimilation	Transparent	Opaque
MLT	Malate assimilation	Transparent	Opaque
CIT	Citrate assimilation	Transparent	Opaque
PAC	Phenyl-acetate assimilation	Transparent	Opaque
Oxidase	Cytochrome oxidase	Colourless	Purple

2.7.4 Procedure followed for inoculating API 50CH strip

The strips were opened aseptically and labelled appropriately. Five mL of sterile distilled water was added into the tray to provide a moist atmosphere to prevent the drying of the strip. The strip was laid inside the tray. Suspension for inoculation with turbidity equivalent to 2 McFarland was prepared in ampule of API 50CH. The tubes were then filled with the inoculated API 50CH. After the inoculation the strips were incubated at 29° C for 24 hours. A tube is scored positive test when the colour is changed to yellow by acidification revealed by the phenol red indicator contained in the medium. In case of esculent test (tube no. 25), a change in colour from red to black is scored as positive.

2.7.5 Oxidase test

The ampule containing the oxidase reagent (Oxidase reagent-*bioMérieux*) was placed inside the reusable ampule crusher. The ampule was then crushed opened by gently pressing the middle of the dropper. The reagent was then dispensed (precisely 1 drop) onto a non-impregnated disc (diameter 6 mm). A well-isolated colony from a fresh (18 to 24 hours culture) culture was spotted onto the disc and was observed for colour change. Isolates were segregated as oxidase positive when the colour changed to violet to purple colour within 10 to 30 seconds.

2.8 Antibiotic sensitivity test using disc agar diffusion technique

Overnight cultures of bacterial isolates were adjusted to McFarland opacity 0.5 before inoculation onto Nutrient Agar plates with sterile glass spreader. After 10 to 15 minutes, antibiotic rings were laid on top of the inoculated agar surface. Thereafter, the plates were then inverted and incubated

within 15 minutes of disc application at 30°C for 16–18 hours. The negative control plate was observed for semi-confluent growth in the absence of antibiotic and measurements of the zones of inhibition to the nearest millimetre were taken with a scale (Kirby *et al.,* 1966; Dworkin & Falkow 2006). For each antibiotic present on Mastring14 and Mastring13, sensitivity scores were categorized as greater than zero or exactly zero. Antibiotics that display 0 mm zones of inhibition indicate the organism's resistance to metabolize the exposed antibiotic, whereas those display zones of inhibition greater than zero, indicates the organism's sensitivity to that particular antibiotic. SPSS version 20 and excel 2011 were used to analyse the data.

2.9 Determination of minimum inhibitory concentration for bacterial species against antibiotics

2.9.1 Minimum inhibitory concentration assay by tube dilution technique

The Minimum inhibitory concentration assay by tube dilution technique as conducted as per the protocol of (Chitwood, 1969). The test strains were grown overnight on Luria-Bertani (LB, Oxoid, CM 1018) agar at 37°C. Concentrated stock solutions of all the drugs were prepared at 256µg/mL. 100µl of sterilized LB medium were dispensed into all the wells of a microtitre plate followed by adding 100µl of antibiotic into the wells and subsequent mixing by pipetting up and down. Transferring 100µl from the first well to the second well and so on up to the seventh well did serial dilutions; the eighth wells were used as controls (only medium and no antibiotic). 10µl of each bacterial sample were added to the mixtures, and then incubated at 37°C for 24 hours prior to reading (OD) at 450 nm.

2.10 Identification of bacteria by partial 16S rRNA gene sequence analysis

2.10.1 Bacterial DNA isolation for template preparation for PCR

The bacterial DNA was isolated following the protocol of (Ausubel *et al.*, 2003). 2mL of log phase culture was centrifuged (Eppendorf 5804R) at 5000g for 10 minutes at 4°C. The resulted supernatant was discarded and the cell pellet was dried. The pellet was resuspended in 875µL of Tris EDTA (TE) buffer (pH: 8). 5µL Proteinase K (Sigma- Aldrich) (10mg/mL) and 100µL of 10% SDS was added, mixed gently and incubated at 37°C for 1 hour. Equal volume of phenol-chloroform mixture (1:1) was added to this, centrifuged at 12000g (Eppendorf 5804R) for 10 minutes at 4°C, and then the supernatant was transferred to another microtube. The extraction with phenol-chloroform was done thrice. To the resultant supernatant, 0.1 volumes of 5 M sodium acetate (pH 5.2) and double volume of ice-cold 100% isopropanol were added. This was then incubated at -20°C for 1 hour and was centrifuged at 12000 g for 10 minutes at 4°C (Eppendorf 5804R). The DNA pellet obtained was washed with 70% ethanol, air-dried, and dissolved in 50µL TE buffer (pH.8). The concentration and purity of DNA was estimated spectrophotometrically and appropriate dilutions (~80 -100 ng) were used as template for PCR reactions.

2.10.2 DNA quantification of isolated DNA by spectrophotometer

The DNA was quantified using a UV-Visible spectrophotometer (Thermo spectronic; Genesys.10-S) as per (Sambrook & Russell, 2001). The spectrophotometric readings were taken at wavelengths of 260 nm, 280 nm, and 230 nm. The absorbance at 260 nm allows calculation of the concentration of nucleic acid in the sample. An absorbance value of 1 at 260 nm corresponds to approximately 50µg/mL for double stranded DNA and 40 ng/µL of RNA. The ratio between the readings at 260 nm and 280 nm (OD260/OD280) indicates the purity of the DNA. Pure

preparation of DNA has OD260/OD280 ratio in the range of 1.8-2.0, or else it indicates protein contamination.

2.10.3 Procedure for DNA quantification

500µL of distilled water was taken in a cuvette and placed in the spectrophotometer (Thermo spectronic; Genesys.10-S). The wavelength was adjusted to 260 and the water was used to autozero the spectrophotometer. Next, 10µL of DNA was diluted in 490µL of distilled water in a cuvette and its absorbance was measured at 260 nm. Each reading was taken in triplicates and their means were used for calculating concentration of DNA using the formula: A_{260} x Dilution factor (50) x 50 = µg/mL

2.10.4 Polymerase chain reaction of 16S rDNA gene using universal primers

Polymerase chain reactions were using a primer pair of 16S rDNA. A portion of the 16S rRNA gene (1.5kb) was amplified from the genomic DNA. PCR reaction mix was made on ice in PCR tubes (Fisher scientific) using commercially available PCR reagents (Promega).

PCR Mix composition

10X PCR buffer	2µL
2 mM each dNTPs	2µL
Forward primer (10 picomoles) (Sigma Aldrich)	2µL
Reverse primer (10 picomoles) (Sigma Aldrich)	2µL
*Taq*DNA polymerase (1U/µL) (Sigma-Aldrich)	1µL
Template DNA (50 ng/µL)	3µL
MgCl2 (Sigma-Aldrich)	1.2µL

Sterile distilled water to a final volume of 20µL

Amplifications were carried out in a Thermal Cycler (C1000™-Biorad) using the following program: a hot start cycle of 94°C for 5 minutes, then 30 cycles of 94°C for 1 minute, annealing at 56°C for 1 minute and extension at 72°C for 90 seconds, ending with a final extension step of 72°C for 10 minutes.

2.10.5 Agarose gel electrophoresis of the isolated DNA for testing integrity

Agarose gel electrophoresis as performed following the protocol of Sambrook & Russell (2001). For the analysis of the amplified products 1% agarose gels were prepared. They were prepared in Tris-Acetate-EDTA (TAE) buffer. Gel Green was added at a final concentration of 0.5mg/mL. The preparation is then melted in a microwave oven and was poured into the gel-casting tray after cooling to 65°C. Appropriate comb was placed within to create well. Once the agarose gel was set, the inserted comb was gently removed and the tray was placed in the electrophoresis tank. Aliquots (5µL) of the amplified PCR products were analysed by electrophoresis. 1% agarose (make) gel containing Gel Green (5 µL of the GelGreen10,000X stock reagent to 50mL agarose gel) was prepared in 1X (Tris-acetate-EDTA) TAE buffer. The DNA sample was mixed with gel loading dye was and loaded into the wells. Electrophoresis was carried out at 80 V for 1 hour (Biorad). The gel was visualized under the transilluminator and gel pictures were captured using Gel documentation system (Biorad).

2.10.6 DNA sequencing of the PCR products of 16S rRNA sequencing

Nucleotide sequences of the PCR amplicon of 16S rRNA gene were determined. The identity was determined by comparing the sequences obtained with the gene sequences available in the Genbank database using Basic Local Alignment Search Tool (BLAST) software (Altschul *et al.*, 1997) at NCBI site (*http://blast.ncbi.nlm.nih.gov. Accessed 20/9/14*).

2.11 Isolation of plasmids from bacterial cells

Plasmid isolation was performed by alkaline lysis method. The method depends on bacterial cell lysis by sodium hydroxide and sodium dodecyl sulfate (SDS), followed by neutralization with a high concentration of low-pH potassium acetate. This gives selective precipitation of the bacterial chromosomal DNA and other high molecular-weight cellular components. The plasmid DNA remains in suspension and is precipitated with ethanol.

Procedure for plasmid isolation by mini prep was carried out according to Kado & Liu 1981; Sathyanarayana & Verma 1993. Briefly, bacterial cultures were grown at 37°C for overnight in 50mL LB broth at 220rpm in an orbital shaker incubator (Stuart.S150) until they reached an OD of 1.7 at 600 nm (Thermo spectronic; Genesys.10-S). 15mL of each culture was harvested by centrifugation at 14000rpm for 15 minutes at 4°C (Eppendorf 5417 R). The bacterial pellet was then resuspended in 250µL of resuspension buffer (50 mM TrisHCl, pH 8.0, 10 mM EDTA) and 100µg/mL RNase A, and stored at 4°C until required. This was followed by membrane solubilisation process, which was done by adding 250 µl of lysis buffer (200 mM NaOH, 1% sodium dodecyl sulphate (SDS); stored at room temperature not longer than one week). The sample was then mixed gently by inverting the tube several times and was then incubated at room temperature for 60 minutes. Then, 350µL of neutralization buffer (3.0 M Potassium acetate, pH 5.5) was added to each solution and then mixed gently by inverting six times, then centrifuged at 14000rpm for 15 minutes (Eppendorf 5804 R). The clear supernatant was then transferred into a new microfuge tubes containing 300µL of 1:1 phenol: chloroform solution. This was mixed thoroughly and then centrifuged at 16000rpm for 15 minutes at 4°C. The supernatant was carefully transferred into a fresh tube without disturbing the bulky interphase. The clear supernatant was taken and the plasmid DNA was precipitated by adding

600µl of isopropanol followed by centrifugation at 16000rpm for 15 minutes at 4°C. The resulting pellets were washed with 70% ethanol and precipitated by centrifugation at 16000rpm (Eppendorf 5804 R) for 2 minutes at 4°C. The pellets were then dried and re-suspended in 60µL TrisHCl, pH 8.5 buffers or in double distilled water and stored in refrigeration at -20^{0}C.

2.11.1 Preparation of competent cells for transformation experiments

E.coli DH5α was used for transformation and competent cell preparation. Competent cells were prepared following the procedure of (Sambrook & Russell, 2001). A single bacterial colony of *E.coli* DH5α was inoculated into 100mL LB broth and incubated at 37°C in a 1 L flask with vigorous agitation. The bacterial culture was transferred to ice-cold centrifuge tubes and the tubes were kept on ice for 10 minutes. The culture was centrifuged at 4000g for 10 minutes at 4°C (Eppendorf 5804 R). The supernatant was decanted and the tubes were kept inverted for 1 minute. The pellet was resuspended in 10mL of ice-cold 0.1 M $CaCl_2$ solutions by gentle vortexing and stored on ice for 10 minutes. The cells were recovered by centrifugation at 4000 g for 10 minutes at 4°C (Eppendorf 5804 R). The medium was decanted and the tubes kept inverted for 1 minute. The pellet was resuspended by gentle vortexing in 2mL of ice-cold 0.1 M $CaCl_2$ for each 50mL of original culture. The cells were then used immediately or were stored at -80°C.

2.11.2 Transformation of competent *E.coli* cells with the isolated plasmids

Bacterial transformation was performed using 1µL of pUC19 vector (without insert) and 5µl of pUC19 (with insert) using DH5α^{TM} competent *E. coli* cells. Bacterial transformation was done by temperature heat shock method as per the procedure of (Sambrook & Russell, 2001). Frozen ^{TM}DH5α^{TM} competent *E. coli* cells maintained at -80°C were taken out of freezer and

immediately placed on ice. Cells were allowed to thaw slowly on ice and in the meantime the heat block was set to 42°C (1.5mL Eppendorf Thermomixer comfort).

After thawing, 25µL of competent cells were transferred to pre-chilled and pre-labelled 1.5 mL Eppendorf tubes. Then 2µl (10ng) of each of the plasmid was then added to each of the two tubes containing the 25µL of the competent cells. Two pre-labelled tubes were prepared as controls, one using 1µl of sterilized water as the negative control and another 1.5 µl of pUC19 plasmid as the positive control. After gentle mixing, the tubes were then placed back on ice and allowed to incubate at 0°C for 30 minutes. After incubation, the tubes were then placed on the heat block maintained at 42°C as a heat shock for 45 seconds during which transformation should occur. The tubes were then placed back on ice for 2 minutes and then at room temperature. Pre-heated LB Broth at 37°C was taken out of a water bath and 500µL of the broth was gently added to the transformation tubes. The tubes were then placed in the shaking incubator (Stuart. S150) at 37°C and was incubated for 60 minutes at 225 revolutions per minute (rpm).

LB-agar plates were made with varying concentrations of ampicillin (16, 32, 64 and 128µg/mL), 100µL of the mixture from the transformation tubes was carefully transferred on to duplicate LB-agar plate and spread evenly with the help of a sterile glass spreader. The plates were then incubated overnight at 37°C to allow the transformed bacteria to form colonies.

2.12 Screening for efflux pump mediated antibiotic resistance in bacterial species

The screening assay was carried out as per the protocol of (Malek *et al.*, 2009). The test strains were grown overnight on Luria-Bertani (LB, Oxoid, CM 1018) agar at 37°C. LB broth with varying concentration (5-128µg/mL) ampicillin and tetracycline where prepared in two sets. One set was without (carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) and one set with CCCP at final concentration of 15µg/mL. These test tubes were then subsequently inoculated with the test

organisms. The cultures were then incubated at 37°C for 24 hours. The MIC for ampicillin and tetracycline of different isolates were determined. The test was conducted in the absence and presence of CCCP as internal controls.

2.13 Determination of penicillinase activity in bacterial species

2.13.1 Phenol red indication method for detecting penicillinase activity

One minute tube test (Escamilla, 1976) was performed by mixing phenol red solution with penicillin. This was then scarped over the bacterial cultures. Results of this method can be determined based on the colour change. The development of yellow colour is an indication of penicillinase production.

2.13.2 Tube dilution test for detecting penicillinase activity

The penicillinase activity test was carried out using varying concentration of ampicillin with and without 16ug/mL of clavulanic acid. The test strains were grown overnight on Luria-Bertani (LB, Oxoid, CM 1018) agar at 37°C. LB broth with varying concentration (16-128µg/mL) ampicillin prepared in two sets. One set was without clavulanic acid and one set with clavulanic acid at final concentration of 16µg/mL. These test tubes were then subsequently inoculated with the test organisms. The cultures were then incubated at 37°C for 24 hours. The MIC for ampicillin and tetracycline of different isolates were determined. The test was conducted in the absence and presence of clavulanic acid as internal controls.

2.14 Outer membrane protein extraction of selected bacterial species

The outer membrane proteins were extracted as per the protocol of (Hobb *et al.,* 2009). The bacterial suspensions were centrifuged at 5000rpm for 20 minutes (Eppendorf 5804R). The cells were then washed thrice in 25mM/l Tris buffer (pH 7.4) containing 1 mM$MgCl_2$ followed by

homogenization (Jencons 361-044) for 2.5 minutes in ice. Sarcosine (Sigma 131776) was added to a final concentration of 2% (w/v). The samples were then kept on ice for 20 minutes for precipitation of the proteins. The insoluble outer membrane was then sediment by centrifugation at 13000rpm for 1 hour at 4°C (Eppendorf 5417 R). The samples were stored in -80°C until use. For SDS-PAGE the pellets were resuspended in 50mM of Tris HCl (100µl) (pH 6.8). The protein samples were diluted 1:1 with SDS buffer.

2.14.1 Protein estimation of the outer membrane protein

Protein concentration was calculated according to the method of (Bradford, 1976) using bovine serum albumin (BSA) as the standard and the concentration was expressed in mg/mL. Standard graph was plotted and the slope was calculated. Two points were chosen to find the y intercept. The slope of the standard graph was calculated using the equation (y = mx + b). **Y** is the absorbance, **m** is the slope, and x is the concentration. The protein concentration of the samples was determined from the slope. The absorbance of the sample is divided by the value of the slope and the concentration is obtained. The value is adjusted by multiplying by the dilution factor.

2.14.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) of outer membrane proteins

The gel plates were cleaned and assembled. Resolving gel (10%) was prepared by mixing 10mL of acrylamide: bis-acrylamide (30:0.8), 3.75mL of resolving gel buffer stock, 300µL of 10% SDS and 15.95mL of water .The solution was degassed and 100µL of ammonium persulfate solution (10%) and TEMED (15µL) were added. The mixture was immediately poured into the cast and a layer of water was added over the gel and allowed to polymerize for at least one hour.

Table 2.3: Stacking gel and separating gel composition for SDS PAGE			
Stacking gel		**Separating gel**	
Gel %	4	Gel %	12
30% polyacrylamide (mL)	17	30% polyacrylamide (mL)	60
1mM Tris (pH 6.8) (mL)	12.5	1mM Tris (pH 6.8) (mL)	37.5
10 % Ammonium persulphate (APS) (mL)	1	10 % Ammonium persulphate (APS) (mL)	1.5
10% SDS (mL)	0.1	10% SDS (mL)	1.5
Water	68	Water	49.49
TEMED (mL)	0.1	TEMED (mL)	0.06
Total volume (mL)	100	Total volume (mL)	150

Water layer was poured out after polymerization. The stacking gel (2.5%) was prepared by combining 2.5mL of 30: 0.8 acrylamide: bis-acrylamide solution, 5mL of stacking gel buffer stock, 200µL 10% SDS and 12.3mL of distilled water, followed by 100µL of ammonium persulfate and 15µL of TEMED. The stacking gel was then poured into the gel assembly, above the resolving gel and the comb was immediately inserted. Gel was allowed to polymerize for 30 minutes, placed in the electrophoresis apparatus and upper and lower reservoirs filled with reservoir buffer and was pre run for 1 hour at 80 V. Sample was prepared by mixing 100µL of 1X sample buffer with protein sample. 25µL of this sample and 5µL low molecular weight marker mix was loaded on to the gel and run at 80 V. The current was increased to 100 V, when the dye front entered the resolving gel. The run was stopped when the dye front reached 1 cm from the lower end of the plate, the gel was removed and stained.

2.14.3 Coomassie blue staining of the SDS PAGE gel with outer membrane protein

After the electrophoresis run, the gel was immersed in a medium size microwave container with Coomassie blue stain (1 g of Coomassie Brilliant Blue in 1 litre of solution containing: Methanol (50% [v/v]), Glacial acetic acid (10% [v/v]) H_2O (40%) filtered through Whatman filter paper). The container was then placed in a microwave oven at the highest power for 1 minute. After that

the gel was further incubated at room temperature for 5 minutes. The gel was then rinsed thoroughly in tap water and was destained in super destain solution (methanol, and acetic acid in a ratio of 50/40/10 (v/v/v). The destaining was carried out inside the microwave at the highest power for 1 minute. The destaining was continued up to 15 minutes after the removal from microwave oven and the destaining solution was replaced with fresh one. The incubation was continued up until the bands appeared at around 40 minutes.

2.14.4 Silver staining of the SDS PAGE gel with outer membrane protein

The silver staining of SDS PAGE gels were carried out as per the protocol of (Blum *et al.,* 1987). The gel was fixed for 30 minutes in the fixing solution 1 (50mL methanol and 5mL acetic acid in 45mL water), followed by incubation in fixing solution 2 (50mL methanol in 50mL water) for 15 minutes. This gel was washed 5 times in water for of 5 minutes each. The gel was then sensitized with freshly prepared sensitizer Sodium thiosulphate (20mg/100mL) for 1 minute and was rinsed in water for two times for 2 minutes. It was then incubated in freshly prepared staining solution silver nitrate (200mg/100mL) for 25 minutes at 4°C, following by washing two times for 1 minute each. The gel was then incubated in freshly prepared developing solution (Sodium carbonate (anhydrous) - 3g/100mL, Formaldehyde - 25µL/100mL and Sodium EDTA solution - 1.4 g/100mL) until the bands appeared. In order to prevent over-staining, the gel was treated for an additional 10 minutes with sodium EDTA solution and was washed in water for twice for 2 minutes. The photo-image of the gel was taken using gel documentation system (gel doc 100, Bio Rad, Hercules USA). The images were analysed using Band scanner analyser (5.1 software).

2.14.5 Molecular weight determination of outer membrane protein separated on SDS PAGE gel

The molecular weights of separated proteins are determined by measuring the relative migration distance (*Rf*) of the protein standards and the test protein.

The migration distance can be calculated using the following formula:

$$Rf = \frac{\text{Migration distance of the protein (mm)}}{\text{Migration distance of the dye front (mm)}}$$

A standard curve was plotted using the logarithm of the molecular weight of standard protein markers and its corresponding *Rf* (Figure 5.5). A linear trendline was applied and the graph equation was calculated as $y = -2.3855x + 2.2647$. Molecular weight of outer membrane proteins were then calculated using equation $MW = 10^y$, where x = *Rf* of unknown protein. By substituting Rf value (x) of each bands to the equation, $MW=10^{-2.3855x + 2.2647}$, the molecular weight is calculated.

2.15 RNA extraction by TRIZOL method from selected bacterial species

The RNA extraction was carried using TRIZOL reagent as per the protocol of (Hongbao *et al.*, 2008). An aliquot (10mL) of each bacterial culture was grown overnight at 37°C at 220rpm in a shaking incubator (Stuart.S150). The bacterial cells were then sediment via by centrifugation at 8000rpm for 10 minutes (Eppendorf 5417 R). The cells were then washed in DNase- and RNase-free distilled water twice to avoid the degradation of the fragile mRNA. The bacterial cells were then lysed in 1mL TRIZOL reagent by repetitive pipetting. A homogenizer was used to disrupt the bacterial cells to aid in release of mRNA. The mixture was then incubated at room temperature for 5 minutes to permit complete dissociation of the nucleoprotein complex. After

the incubation, 0.2mL of chloroform/1mL was added to it. The tubes were then shaken vigorously by hand for about 15 seconds and were incubated at room temperature for another 3 minutes. After the incubation, the tubes were centrifuged at 12000g for 15 minutes at 4°C (Eppendorf 5417 R). The RNA gets collected in the top aqueous phase. This was then transferred to a fresh tube. The isolated RNA was then precipitated from aqueous phase by the addition of 0.5mL of 100% isopropanol (isopropyl alcohol, C_3H_8O) solution. After the addition of isopropanol the tubes were incubated for 10 minutes at room temperature. The resultant precipitate contains RNA. The RNA is then harvested by centrifugation at 12000g for 10 minutes at 4°C (Eppendorf 5417 R). The isolated RNA forms a gel-like pellet at the bottom. The supernatant was removed and the RNA pellet was washed once with 75% ethanol and air-dried for 5-10 minutes. The pellet was then dissolved and mixed in 0.03–0.06mL RNase-free water or 0.5% SDS solution followed by incubation for 10 minutes at 55-60° C. This was then re dissolved in 100% formamide (deionized).

2.15.1 Purification of total after RNA extraction using RNeasy Mini Kit and DNase digestion by RNase- free DNA set

Purification of total RNA from bacterial lysate was done using the RNeasy Mini Kit and DNase digestion was performed using QIAGEN DNase digestion by RNase- free DNA set following the manufacturer`s instruction. Briefly an aliquot (700µL) of each crude RNA lysate was transferred into minispin columns and was placed into 2mL collection tube. This was then centrifuged at 8000g for 15 seconds (Eppendorf 5417 R) and the flow through was discarded. Presence of trace quantities of DNA in the RNA preparation may hinder RT-PCR analysis. DNase digestion was carried out to remove the contaminating DNA. For DNase digestion an aliquot (20µl) of DNase 1 solution was mixed with 140µL Buffer RDD by gently inverting the tube. The residual liquid

from the sides of the tube was then collected by brief centrifugation at 1000g for 1 minute (Eppendorf 5417 R). This DNase incubation mix was then added to the RNeasy spin column membrane and was incubated at room temperature. After the incubation 350µl of RW1 was added to the RNeasy spin column. The columns were centrifuged at 8000g for 15 seconds (Eppendorf 5417 R) to wash the spin column membrane. This was followed by addition of 500µl RPE buffer and another round of centrifugation at 8000rpm for 15 seconds (Eppendorf 5417 R). The flow through was then discarded and the process was repeated with 500µl of RPE buffer. Finally 500µl of RNase free water was added to each sample and was centrifuged for 1 minute. The flow through was discarded. An aliquot (700µl) of RW1 buffer was added to the spin column. The column was then washed by centrifugation at 8000g for 15 seconds (Eppendorf 5417 R). The spin columns were replaced in fresh 2mL collection tubes and 500µL of RPE buffer was added. This was followed by one more round of centrifugation at 8000g for 15 seconds. The resulting flow through was discarded and 500µl of RPE buffer was added followed by 2 minutes of centrifugation at 10000rpm (Eppendorf 5417 R). The columns were then placed in new collection tubes and 50µl of RNase free water was added and as centrifugation at 8000rpm for 1 minute (Eppendorf 5417 R) to elute the purified RNA.

2.15.2 Procedure for RNA quantification by spectrophotometer

RNA dilution buffer (10mM TrisHCl, pH 7) was taken in a sterile cuvette and placed in the spectrophotometer (Thermo spectronic; Genesys.10-S). The wavelength was adjusted to 260 and the buffer was used to blank the spectrophotometer. Next, 10µL of RNA was diluted in 490µL; of RNA dilution buffer in a cuvette and its absorbance was measured at A260nm. Each reading was taken in triplicate and concentration of RNA was calculated using the formula.

A_{260} x Dilution factor (50) x 44 = µg/mL

2.16 cDNA synthesis using total RNA extracted from selected bacterial species

cDNA synthesis was carried using the extracted and purified RNA. The integrity and purity of the isolated total RNA was confirmed using spectrophotometer (Thermo spectronic; Genesys.10-S). cDNA synthesis was performed using a commercially available kit - RT2 First Strand Kit – Qiagen.

2.16.1 Procedure for cDNA synthesis using RT2 First Strand Kit, Qiagen

Six µl of proprietary Buffer GE (gDNA elimination buffer) was mixed with 5µg of RNA. The reaction mixture was made up to 14µl using RNase-free H2O. The mixture was incubated at 37°C for 5 minutes and was immediately placed on ice for at 1 minute. After the incubation 6µl of the Reverse Transcriptase Mix was added and the mixture was incubated at 42°C for 15 minutes. The reaction was immediately stopped by heating the reaction mixture at 95°C for 5 minutes. Once the cDNA synthesis is over the product is stored in ice until commencement of PCR.

2.16.2 Designing of primers for virulence gene screening

Four pairs of primers were designed using the conserved stretches of the respective virulence genes that are already reported and available in the NCBI database. Primer designing was done using Primer3 software (Primer3, n.d.). The specificity of the primers was confirmed by the GenBank database Basic Local Alignment Search Tool (BLAST) program (Altschul *et al.,* 1997) at NCBI site. (http://blast.ncbi.nlm.nih.gov, accessed on 17/03/15). Various properties of the primer including length, GC content, melting temperature, molecular weight and concentration were analysed using Oligonucleotide analyser tool from Integrated DNA technologies (IDT)

(Integrated DNA Technologies 2014). The primer sequence and the expected amplicon sizes are as given in **Table.5.1** of **Chapter 5.**

2.16.3 Polymerase chain reaction using cDNA synthesized to screen and quantify virulence gene

Polymerase chain reaction was using several primers (**Table 5.6** of **Chapter 5**). PCR reaction mix was made on ice in PCR tubes (Fisher scientific) using commercially available PCR reagents (Sigma- Aldrich). The primers were diluted from the standard to required working concentration accordingly. Amplifications of cDNA were carried out in a Thermal Cycler (C1000 touch thermal cycler, BioRad) using the following program: a hot start cycle of 94°C for 5 minutes, then 30 cycles of 94°C for 1 minute, annealing at 59°C for 1 minute and extension at 72°C for 90 seconds, ending with a final extension step of 72°C for 10 minutes.

PCR mix composition

Go Taq® Flexi Buffer	3µL
$MgCl_2$ (25mM)	3µL (final concentration 2.5mM)
Forward Primer (5uM stocks)	3µL (final concentration 0.5uM)
Reverse Primer (5uM stocks)	3µL (final concentration 0.5uM)
dNTP mix (2mM each)	3µL (final concentration 0.2mM)
DNA template	50ng
Go Taq® DNA polymerase	1 unit
Nuclease Free water	up to 30µL

5µL of the PCR product samples were analysed by electrophoresis on 1.5 % agarose. The PCR was performed in triplicates for each of the strain and each of the genes used.

2.16.4 Agarose gel electrophoresis of PCR products with virulence genes

For the analysis of the amplified products 1% agarose gels were prepared. They were prepared in Tris-Acetate-EDTA (TAE) buffer. Gel Green was added at a final concentration of 0.5mg/mL. The preparation is then melted in a microwave oven for a minute and was poured into the gel-casting tray after cooling to 65°C. Appropriate comb was placed within to create well. Once the agarose gel was set, the inserted comb was gently removed and the tray was placed in the electrophoresis tank. Aliquots (5µL) of PCR product was mixed with gel loading dye and loaded into the wells. DNA markers (1 kb marker) were run along with the products. Electrophoresis was performed at a constant volt (5V/cm) and gel pictures were captured using Gel Doc 100 (Bio-Rad, Hercules, USA) and the images were analysed by using Band scan analyser 5.1 software.

Chapter 3. Isolation and Identification of Bacterial Isolates with Multi-drug Resistance from the Dighty Burn

This chapter addresses the experimental processes designed to isolate and identify antibiotic resistant allochthonous bacterial strains from the Dighty Burn. The Chapter includes an introduction (Section 3.1), the experimental approach (Section 3.2), results (Section 3.3), discussion (Section 3.4) and a chapter summary and conclusions (Section 3.5).

3.1 Introduction

Rivers and streams have been shown to be major sources of antibiotic resistance in the environment. These freshwater bodies act as reservoirs for all possible anthropogenic inputs that contribute to the development of antibiotic resistance in environmental bacteria. Examples of anthropogenic inputs include waste water treatment plant effluent, which increases the prevalence of clinically important resistant bacteria and resistant genes(Amos *et al.*, 2014; Amos, *et al.*, 2014) agricultural pollution where antibiotic resistant bacteria reach the environment via animal faeces and slurry application (Byrne-Bailey *et al.*, 2009; Chee-Sanford *et al.*, 2001; Kay *et al.*, 2005) and finally, detergent found on industrial effluents that co-selects for Class 1 integrons (Gaze *et al.*, 2005). The classes 1 integrons are genetic elements that routinely contain mobile antibiotic and biocide-resistance genes (Stokes & Hall, 1989), which have been found in a wide range of polluted environments, such as sewage-sludge-amended soil (Gaze *et al.*, 2011) and waste water treatment effluent (Stalder *et al.*, 2014). Class 1 integrons are capable of integrating gene cassettes into a variable region and to date, there are 130 known gene cassettes conferring a range of antibiotic-resistant phenotypes, thus Class 1 integrons equip bacteria with the ability to become resistant to a wide range of antibiotics (Partridge *et al.*, 2009).

This chapter investigates the processes of bacterial isolation, identification, and characterization studies of environmental bacteria in order to assess the prevalence of antibiotic resistance in this river environment. Samples were collected using the same procedure described for monitoring and providing special natural characteristics of watercourses as rivers (JNCC, 2014).

3.2 Experimental approach

The method used for water sampling and analysis as recommended by several authoritative bodies is the Membrane filtration (MF) technique. This technique is widely used in standard bacteriological tests to assess water quality. Membrane filtration utilizes a physical barrier, a sterile porous membrane or a filter, to separate particles from a liquid. Particles and microorganisms are separated based on their size and shape with the use of pressure and specially designed membranes of different pore sizes. Microorganisms retained in the filter can then be grown on agar medium to produce visible colonies that can be isolated and further identified. One of the advantages of the MF technique is that it allows large sample testing with little preparation time compared to other analysis options. One of the drawbacks of membrane filtration is that it cannot be used to assess water quality from a turbid water source. However, this was not an issue for this experiment, since samples were obtained from a clear water source.

3.2.1 Membrane filtration technique

The technique involves collection and examination of bacteria on sterilized gridded cellulose-acetate membrane filters of 47 mm diameter with pore size 0.22μM (Advantec MFS, Inc). Filters were placed either directly on the nutrient agar or pads impregnated with agar broth for

incubation at temperature 25°C or 37°C. After incubation the membrane filters were removed and the agar plates were examined for growth of colonies. The detailed methodology followed for membrane filtration of the Dighty river water sample is as given in **Chapter 2**, section **2.2.**

3.2.2 Bacterial enumeration by viable plating techniques

In order to determine the extent of bacterial load, counting of viable cells in a sample is a standard procedure. The viable microbial cell count measures the concentration of living microorganisms present in the sample. The widely accepted definition of cell viability is the ability to multiply and produce either a macroscopic colony on an appropriate solid growth medium or generate visible turbidity in an appropriate culture (Heritage, 2011). The spread-plate technique is used for the separation of dilute, mixed population of microorganisms so that individual colonies can be isolated (Aneja, 2003). Each colony forming unit (CFU) represents a single bacterium in the original diluted sample (Kapoor & Yadav, 1986). Colonies appearing on the medium in a plate are visible to the naked eyes and can be counted. For better results, the original sample must be diluted so that an optimum number of colonies between 30 and 300 are obtained on the medium (Heritage, 2011). It has been observed that fewer than 30 colonies make the interpretation of results statistically insignificant and greater than 300 colonies leads to overlapping colonies that leads to skew and imprecise colony counts (Heritage, 2011). The methodology followed for spread plating and quadrant streaking is as described in detail in **Chapter 2**, sections **2.4.1** and **2.4.2**.

3.2.3 Pure culture and identification of selected bacterial isolates

In order to obtain pure bacterial cultures, a total of 400 isolated colonies were sub-cultured in nutrient agar or malt extract agar. Only those colonies that were single and non-overlapping were picked for streaking on another fresh agar plate. These petri plates were incubated having

the same coding system and conditions for aerobic and anaerobic growth and same temperatures (25°C and 37°C) for 48 hours prior to further identification (Clinical and Laboratory Standards Institute., 2007).

3.2.4 Identification of colonies

Gram's staining with subsequent microscopic examination, as well as biochemical identification using Analytical Profile Index (API), was conducted to identify the purified bacterial isolates. Identification procedures were then undertaken using the protocols of (Breed *et al.*, 1957)

3.2.4.1 Gram's staining

The Gram stain is a differential staining technique used to classify and categorize bacteria into two major groups, Gram-positive and Gram-negative. The methodology followed for performing Gram staining is as given in **Chapter 2**, section **2.6.1.**

3.2.4.2 Selective and differential culture media

MacConkey Agar (Oxoid, CM0007) was used for the isolation of Gram-negative enteric bacteria and for the differentiation of lactose-fermenting from lactose non-lactose-fermenting Gram-negative bacteria. Bile salts and crystal violet inhibit the growth of Gram-positive bacteria. Lactose provides a source of fermentable carbohydrate, allowing for differentiation. Neutral red is a pH indicator that turns red at a pH below 6.8 and is colourless at any pH greater than 6.8. Lactose-fermenting organisms will grow as pink colonies with or without a zone of precipitated bile. Non-lactose fermenting organisms grow as colourless or clear colonies.

3.2.4.3 Preliminary identification process with API strips

The analytical profile index (API) is a detection system for bacterial classification and identification of closely related bacteria. There are more than 15 kinds of commercially available strips formulated to classify unidentified bacterial isolates up to the species level. Different test panels are prepared in dehydrated forms, which are reconstituted upon use by addition of bacterial suspensions. The most common strips used for identification and classifications of Gram-negative bacteria are API 20E and API 20NE. API 20E is used for the identification of enteric Gram-negative bacilli, while API 20 NE is a standardized system for the identification of non-fastidious, non-enteric Gram-negative rods. API 50CH is the corresponding API strip system used for identifying and classifying Gram-positive bacteria.

In order to further identify the purified cultures using the API strips systems, based on the Gram staining and MacConkey experiments 163 Gram-negative strains and 47 Gram-positive bacterial isolates were grown overnight at 37°C in nutrient broth medium. Bacterial suspensions were compared side by side with McFarland as the API manufacturer recommended the determination of the appropriate turbidity of the inoculum. The API strips were then inoculated onto a saline suspension containing the pure culture and then covered with plastic impermeable container with a little sterile water inside it to avoid evaporation. After 24 hours of incubation in a humidity chamber at 37°C, colour reactions were developed as a result of rehydration reaction occurring inside each test compartments as well as due to changes in pH. Colour complexes can be developed directly either by chemical reaction with other chemicals present in the tube or by manual addition of special reagents. The results for each strip were recorded and analysed using the "APIweb" database (http://www.biomerieux-usa.com/clinical/api, Accessed March 9, 2016). The methodology followed for handling and inoculating the API strip is discussed in detail in **Chapter 2**, section **2.7**.

3.2.4.4 Oxidase test

The oxidase test is a biochemical reaction used to check the presence of cytochrome oxidase enzyme in bacteria that catalyses the oxidation of cytochrome C while reducing oxygen to water. In the presence of atmospheric oxygen and cytochrome C, the enzyme oxidizes the phenylenediamine reagent to form a purple coloured compound, indophenol. The procedure followed for conducting the oxidase test is described in **Chapter 2**, section **2.7.5.**

3.2.5 Determination of antibiotic sensitivity using the Mastring assay

MASTRING-S™ is an antibiotic susceptibility ring device, designed for convenient and simultaneous testing of an equivalent of 6 or 8 antibiotics. The Mastring is composed of a filter paper circular ring with several attached peninsulae or tips. Each tip is impregnated with a different antibiotic and functions as an individual antibiotic sensitivity disc. Two different combinations of antibiotic disc rings were used in this study, M13 and M14 (MAST Diagnostics Co., France). M13 contains the stock of rings of antibiotics for testing antibiotic susceptibility of Gram-positive bacteria and M14 contain susceptibility rings for Gram-negative bacteria. Refer to **Chapter 2**, section **2.8** for the detailed procedure followed for antibiotic sensitivity using the Mastring assay.

3.2.6 Statistical analysis

SPSS version 20 was used to analyse the experimental data. Statistical Analysis tools used to process the data were determination of the Mean, Standard Deviation, and subsequent t-test to determine if the scores for the two categories have significant differences with each other. The Independent Samples T-Test table consists of the following: N shows the number of species belonging to that group. Mean shows the average sensitivity scores. SD stands for Standard

Deviation and it shows the spread of the data with respect to the Mean (the higher the SD, the wider the data and vice versa). df stands for degrees of freedom. It is computed by subtracting 1 to the N. This is used for Inferential Analysis in determining significant differences between the categories (greater than 0 and those who are exactly 0). T value is a value in the Independent Samples T Test, which tests the significant differences between the categories. P-value indicates the probability that the two categories are not significantly different. A rule is that a p-value of less than 0.05 indicates that there is a significant difference between the two categories. A higher p-value indicates that the two categories are not significantly different.

3.2.7 Determination of minimum inhibitory concentration (MIC)

Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that can inhibit microbial growth (Gharehbeglou *et al.*, 2015). MIC values are frequently used by diagnostic laboratories to confirm specific microbial resistance against an antimicrobial agent and are used as an *in vitro* research tool in many university laboratories (Christofilogiannis, 2001). Standardised methods of performing MIC test either the tube dilution method or the 96 well plate method. In this study, all the bacterial strains demonstrated significant results with both M 13 and M14 Mastrings were subsequently tested with the tube dilution method or the 96 well plate method in order to determine their antibiotic sensitivity determined as MIC. The procedure for carrying out tube dilution test in microtitre plate is as given in **Chapter 2**, section **2.9.1**.

3.2.8 Oxidase test

Following the MIC test, the selected strains were subjected to oxidase test. The strains were selected based on their exhibited antibiotic resistance via the 96 well plate test (Clinical and Laboratory Standards Institute., 2007).

3.2.9 Identification of bacteria by 16s rDNA

For rRNA gene sequencing, two primer pairs were used (**Table 3.1**) to amplify 16s rRNA gene in Gram-negative bacteria using PCR (Greisen *et al.*, 1994). After the purified PCR products of the 16s rRNA genes were obtained, they were processed for sequencing. The resulting DNA sequences were aligned against bacterial DNA gene database to identify the bacterial isolates. Refer to Chapter 2, sections 2.10.1, 2.10.4, 2.10.5 and 2.10.6 for the methodology followed for bacterial DNA isolation, 16s rRNA gene PCR, agarose gel electrophoresis and DNA sequencing/analysis respectively.

Table 3.1: Two sets of universal bacterial primers (forward and reverse) were selected as broad range and were used for 16s rRNA gene amplification of the genomic DNA of selected bacteria species (Greisen *et al.*, 1994).

Universal Primers	Sequences	Reference
DG74	5'-AACTGGAGGAAGGTGGGGAC-3'	Greisen *et al.*, 1994
RW01	5'-AACTGGAGGAAGGTGGGGAT-3'	
RDR080	5'-AACTGGAGGAAGGTGGGGAC-3'	
PL06	5'-GGTTAAGTCCCGCAACGAGCGC-3'	

3.3 Results

3.3.1 Isolation and preliminary identification of bacteria

In this study, attempts were made to isolate antibiotic resistant bacteria from Dighty River using membrane filtration technique. **Table 3.2** shows the finer details of the growth conditions used

and observations of the experiment.

The isolated colonies generated from the membrane filters were then propagated in agar media and used to further identify the microbial constituents present in the water samples. For bacterial identification, Gram's staining was initially performed to identify Gram-positive and Gram-negative bacterial isolates. For this experiment, 100 colonies were chosen from each agar plate and grown at 25°C for 48 hours. Overall, a total of 400 colonies were selected for further study.

Table 3.2: Details of the culture parameters used in the isolation of bacteria using membrane filtration technique. Details of growth medium, specific growth conditions (temperature, duration and aerobic/anaerobic) and the observations after 48h are given.

Filters number	Agar Medium	Growth Conditions	Observation
1	Nutrient Agar (NA) (CM0003)	25°C 48h	Growth
2			
3			
4			
5			
6	Malt Extract Agar (MEA) (CM0059)	25 °C 48h	Growth
7			
8			
9			
10			
11	Nutrient Agar (NA) (CM0003)	37 °C 48h (Aerobic)	Growth
12			
13			
14			
15			
16	Nutrient Agar (NA) (CM0003)	37 °C 48h (Anaerobic)	Growth
17			
18			
19			
20			

Then each isolate was further identified up to the species level using biochemical tests and API strips. All the strains that showed as Gram-negative bacteria with the Gram staining technique

were grown and examined on MacConkey Agar prior to further identification using API strips (bioMérieux, France). 88 bacterial cultures that developed pink/red colonies on MacConkey Agar after 24 hours of incubation are lactose fermenters and were classified as non-fastidious, enteric bacteria, e.g. *E. coli*, and labeled for further identification with API 20 E. On the other hand, 75 isolates that displayed transparent, colourless colonies on MacConkey plates were not able to ferment lactose, although they may still be members of the family *Enterobacteriaceae*, e.g. *Salmonella* spp. Non-fastidious, non-enteric, Gram-negative rods (e.g. *Pseudomonas*, *Acinetobacter* etc.) cultures were then examined with API 20NE, a standardized system for the identification based on biochemical tests. API 50CH was used to identify lactobacilli and related genera.

3.3.2 Antibiotic susceptibility test by disc assay

The isolated bacteria were then subjected to antibiotic susceptibility testing using Mastring M13 and M14 and tube dilution methods to determine minimum inhibitory concentration (MIC) of antibiotics effective in inhibiting their growth. Statistical analyses of the results obtained from the Mastring 13 antibiotic sensitivity assay are presented in **Table 3.3.** A total of 146 bacterial isolates were used for this assay. Bacterial cells were uniformly plated on the surface of 100 mm agar plates and exposed to different antibiotics using the M13 ring disc impregnated with eight antibiotic tips containing chloramphenicol (25μg), erythromycin (5μg), fusidic acid (10μg), oxacillin G (5μg), novobiocin (5μg), penicillin G (1 unit), streptomycin (10μg) and tetracycline (25μg). Statistical analyses of mean, standard deviation and *p* values of their zones of inhibition are shown in **Table 3.3**. Results show that out of 146 bacterial isolates tested, 54 were sensitive, while 92 were resistant to chloramphenicol; 16 were sensitive, while 130 were resistant to erythromycin; 24 were sensitive, while 122 were resistant

to fusidic acid; 11 were sensitive, while 135 were resistant to oxacillin G; 18 showed sensitivity, while 128 showed resistance to novobiocin; 13 showed sensitivity, while 133 showed resistance to penicillin G; 101 were sensitive, while 45 showed resistance to streptomycin and 126 were sensitive, while 20 showed resistance to tetracycline. More importantly, differences in the zones of inhibition between the sensitive and resistant strains are all statistically significant indicating that the amount and potency of antibiotics used significantly inhibited the growth of sensitive isolates, while resistant isolates continued to grow without interruption in spite the presence of the drug. Interestingly, **Table 3.4** shows 126 isolates were sensitive, while 20 isolates were resistant to both penicillin G and tetracycline. These 20 resistant strains appear to possess an unknown mechanism that allows them to resist the antibiotic action of both drugs and are good candidates for further analysis for multidrug resistant phenotype.

Table 3.3: Statistical analysis of differences in the size of zones of inhibition (zi) of bacterial isolates that were sensitive (zi > 0) or resistant (zi = 0) to the antibiotics of M13 using Independent Sample T-Test. Where; n = number; SD = standard deviation; df = degrees of freedom; p = probability.

		N	Mean	SD	df	T	p-value	Description
Chloramphenicol	Isolates that were sensitive	54	0.96	0.34	53	20.67	0.00	Significant
	Isolates that were resistant	92	0	0	92			
Erythromycin	Isolates that were sensitive	16	0.8	0.34	15	9.37	0.00	Significant
	Isolates that were resistant	130	0	0	130			
Fusidic Acid	Isolates that were sensitive	24	0.6	0.3	23	9.64	0.00	Significant
	Isolates that were resistant	122	0	0	121			
Oxacillin G	Isolates that were sensitive	11	0.93	0.37	10	8.21	0.00	Significant
	Isolates that were resistant	135	0	0	134			
Novobiocin	Isolates that were sensitive	18	0.66	0.31	17	8.92	0.00	Significant
	Isolates that were resistant	128	0	0	127			
Penicillin G	Isolates that were sensitive	13	0.61	0.28	12	7.96	0.00	Significant
	Isolates that were resistant	133	0	0	132			

Table 3.3 (continued): Statistical analysis of differences in the size of zones of inhibition (zi) of bacterial isolates that were sensitive (zi > 0) or resistant (zi = 0) to the antibiotics of M13 using Independent Sample T-Test. Where; n = number; SD = standard deviation; df = degrees of freedom; p = probability.								
Chloramphenicol		**N**	**Mean**	**SD**	**df**	**T**	**p-value**	**Description**
	Isolates that were sensitive	54	0.96	0.34	53	20.67	0.00	Significant
	Isolates that were resistant	92	0	0	92			
Streptomycin	Isolates that were sensitive	101	0.8	0.33	100	24.22	0.00	Significant
	Isolates that were resistant	45	0	0	44			
Tetracycline	Isolates that were sensitive	126	1.05	0.29	125	39.9	0.00	Significant
	Isolates that were resistant	20	0	0	19			
Total isolates examined against each antibiotic		146	0.9	0.45	125			

Overall, of 146 isolates tested, 129 isolates showed sensitivity to one or more of the eight antibiotics of Mastring 13 group, whereas 17 isolates resisted the action of all antibiotics (**Tables 3.4**). The number of species that are resistant and sensitive towards each antibiotic in Mastring 13 is as given in **Figure 3.1**. A substantial number of species was found to be resistant towards the antibiotics chloramphenicol, erythromycin, fusidic acid, oxacillin G and novobiocin.

Table 3.4: Statistical analysis of differences in the sizes of the zones of inhibition (zi) of bacterial isolates that were sensitive (zi > 0) or resistant (zi = 0) to the combination of the antibiotics Penicillin and Tetracycline using Independent Sample T-Test. Where; n = number; SD = standard deviation; df = degrees of freedom; p = probability.

Penicillin and Tetracycline	N	Mean	SD	df	T	p-value	Description
Isolates that were sensitive	126	0.555	0.19	125	32.669	0.00	Significant
Isolates that were resistant	20	0.00	0.00	19			
Total	146	0.479	0.26	144			

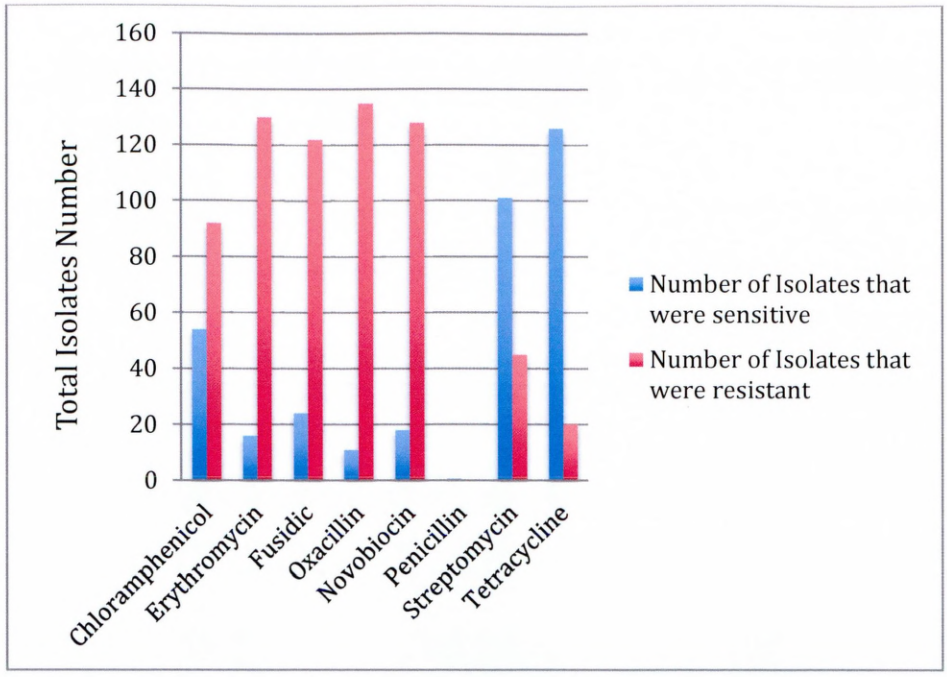

Figure 3.1: Graphical representation showing the number of isolates that are resistant and the number of isolates that were sensitive towards the specific antibiotics Chloramphenicol, Erythromycin, Fusidic acid, Oxacillin G, Novobiocin, Penicillin G, Streptomycin and Tetracycline on the M13 Mastring.

For antibiotic sensitivity assay using Mastring 14 ring disc, statistical analyses of the results are presented in **Table 3.5**. A total of 146 isolates were used for these experiments. All bacterial isolates were uniformly spread on the surface of separate 100 mm agar plates and exposed to different antibiotics using the M14 ring disc impregnated with eight antibiotic tips containing ampicillin (10µg), cephalothin (5µg), colistin sulphate (25µg), gentamicin (10µg), streptomycin (10µg), sulphatriad (200µg), tetracycline (25µg) and co-trimoxazole (25µg). Statistical analysis of mean, standard deviation and *p* values of their zones of inhibition was undertaken. Based on the results, out of 146 bacterial isolates tested, 31 were sensitive, while 114 were resistant to ampicillin; 16 were sensitive, while 130 were resistant to cephalothin; 99 were sensitive, while 47 were resistant to colistin sulphate; 125 were sensitive, while 21 were

resistant to gentamicin; 103 showed sensitivity, while 43 showed resistance to streptomycin; 8 showed sensitivity, while 138 showed resistance to sulphatriad; 139 were sensitive, while 7 showed resistance to tetracycline; and 14 were sensitive, while 122 showed resistance to co-trimoxazole. More importantly, differences in the zones of inhibition displayed by the sensitive versus resistant isolates are statistically significant. Antibiotic-resistant isolates displayed uninterrupted growth with zones of inhibition of zero. This result reveals that the amount and the potency of the antibiotics used significantly inhibited the growth of sensitive isolates, but did not show any significant change in the growth of the resistant isolates. Taken altogether, a total of 143 isolates showed sensitivity to all eight antibiotics belonging to the M14 group, while only 3 isolates showed active resistance to all eight drugs (**Figure 3.2**). These 3 isolates were selected as ideal candidates for further investigation of the multidrug resistant phenotype.

Table 3.5: Statistical analysis of differences in the sizes of the zones of inhibition (zi) of bacterial isolates that were sensitive (zi > 0) or resistant (zi = 0) to all the antibiotics of M14 using Independent Sample T-Test. Where, n = number; SD = standard deviation; df = degrees of freedom; p = probability.

		N	Mean	SD	Df	t	p-value	Significance
Ampicillin	Isolates that were sensitive	31	0.86	0.29	30	16.487	0.00	Significant
	Isolates that were resistant	114	0	0	113			
Cephalothin	Isolates that were resistant	16	0.64	3	15	7.931	0.00	Significant
	Isolates that were sensitive	130	0	0	129			
Colistin Sulphate	Isolates that were sensitive	99	0.79	0.65	48	12.034	0.00	Significant
	Isolates that were resistant	47	0	0	43			
Gentamicin	Isolates that were sensitive	125	0.93	0.4	124	26.08	0.00	Significant
	Isolates that were resistant	21	0	0	20			
Streptomycin	Isolates that were sensitive	103	0.76	0.3	102	17.502	0.00	Significant
	Isolates that were resistant	43	0	0	42			
Sulphatriad	Isolates that were sensitive	8	0.96	0.36	7	7.514	0.00	Significant
	Isolates that were resistant	138	0	0	137			

Table 3.5 (continued): Statistical analysis of differences in the sizes of the zones of inhibition (zi) of bacterial isolates that were sensitive (zi > 0) or resistant (zi = 0) to all the antibiotics of M14 using Independent Sample T-Test. Where, n = number; SD = standard deviation; df = degrees of freedom; p = probability.								
Ampicillin		**N**	**Mean**	**SD**	**Df**	**t**	**p-value**	**Significance**
	Isolates that were sensitive	31	0.86	0.29	30	16.487	0.00	Significant
	Isolates that were resistant	114	0	0	113			
Tetracycline	Isolates that were sensitive	139	0.93	0.33	138	33.53	0.00	Significant
	Isolates that were resistant	7	0	0	6			
Co-trimoxazole	Isolates that were sensitive	14	1.07	0.34	13	11.928	0.00	Significant
	Isolates that were resistant	132	0	0	131			
Total isolates examined against with each antibiotic		146	0.42	0.22	144			

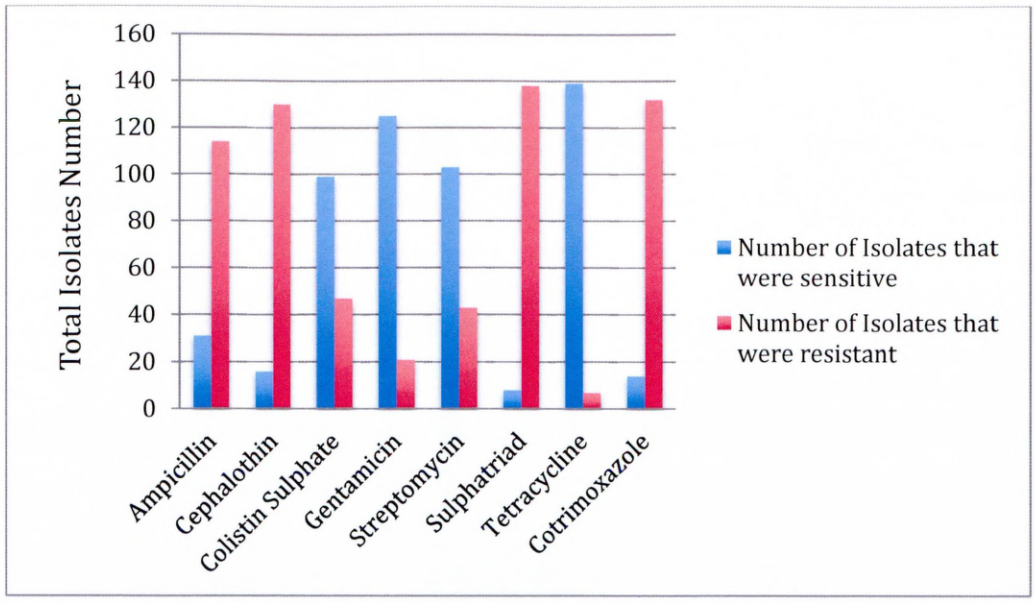

Figure 3.2: Graphical representation showing the number of isolates that are resistant and the number of isolates that are sensitive towards the specific antibiotics Ampicillin, Cephalothin, Colistin Sulphate, Gentamicin, Streptomycin, Sulphatriad, Tetracycline and Co-trimoxazole on the M14 Mastring.

A substantial number of species was found to be resistant towards the antibiotics ampicillin, cephalothin, sulphatriad and co-trimoxazole and sensitive towards colistin sulphate, gentamicin, streptomycin and tetracycline. In addition, with regard to the overall sensitivity, the 146 isolates tested showed higher sensitivity towards the M14 group of antibiotics (with 143 sensitive versus 3 resistant) compared to M13 drugs (129 sensitive versus 17 resistant) (**Figure 3.3**). Finally, a total of 20 putative multidrug resistant isolates confirmed by both M13 (**Figure 3.1**) and M14 (**Figure 3.2**) sensitivity assays were selected along with 13 other promising isolates for further analysis of their antibiotic susceptibility using drug inhibition assay in 96-well plate system.

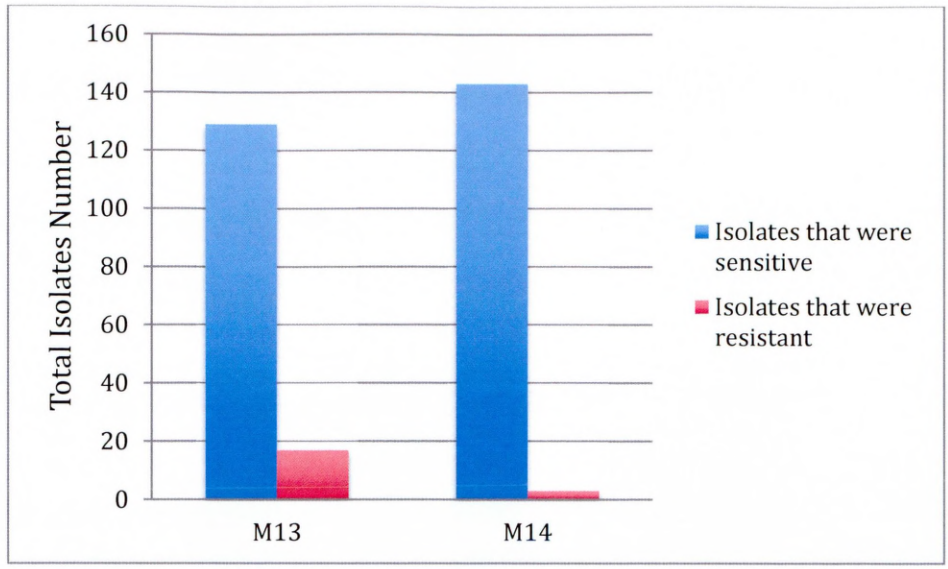

Figure 3.3: Graphical representation showing the number of isolates that are resistant and the number of isolates that are sensitive towards M13 antibiotics (Chloramphenicol, Erythromycin, Fusidic acid, Oxacillin G, Novobiocin, Penicillin G, Streptomycin and Tetracycline) and M14 antibiotics (Ampicillin, Cephalothin, Colistin Sulphate, Gentamicin, Streptomycin, Sulphatriad, Tetracycline and Co-trimoxazole).

3.3.3 Antibiotic susceptibility test by MIC

The antibiotic resistance of bacterial isolates that displayed uninterrupted growth using M13 and M14 were further confirmed using antibiotic susceptibility assay using 96-well plates. **Table 3.6** shows the results of MIC determination of selected antibiotics using the 96-well plates at 450 nm of the preliminarily identified isolate strains. This assay was done in parallel with the Mastring assay to confirm antibiotic sensitivity of selected isolates to four antibiotics of interest, namely ampicillin, co-trimoxazole, novobiocin and tetracycline.

Table 3.6: Determination of Minimum Inhibitory Concentration (MIC; µg/mL) of antibiotics Ampicillin, Clotrimazole, Novobiocin and Tetracycline against preliminarily identified bacterial isolates, using the 96 Well Plate method.

Bacterial Isolates identified by API strips	Ampicillin (µg/mL)	Clotrimazole (µg/mL)	Novobiocin (µg/mL)	Tetracycline (µg/mL)
Aeromonas sobria	>128	> 128	128	2
Vibrio alginolyticus	>128	> 64	64	8
Pseudomonas aureofaciens	> 128	> 128	128	8
Burkholderia cepacia	>128	> 128	128	128
Pseudomonas stutzeri	>128	> 128	>128	>128
Enterobacter spp.	>128	> 128	>128	>128
Citrobacter diversus	>128	> 128	128	32
Enterobacter sakazakii	>128	> 128	64	8
Proteus vulgaris	> 128	> 128	16	8
Enterobacter agglomerans	>128	> 128	>128	>128
Escherichia vulneris	>128	> 128	64	16
Citrobacter freundii	>128	> 128	4	4
Aeromonas hydrophila	>128	> 128	128	128
Listonella damsela	>128	> 128	128	4
Salmonella arizonae	>128	> 128	128	8
Mannheimia haemolytica	>128	> 128	64	8
Chryseomonas luteola	>128	> 128	64	2
Providencia rettgeri	>128	> 128	64	2
Plesiomonas shigelloides	>128	> 128	128	8

Table 3.6 (continued): Determination of Minimum Inhibitory Concentration (MIC; µg/mL) of antibiotics Ampicillin, Clotrimazole, Novobiocin and Tetracycline against preliminarily identified bacterial isolates, using the 96 Well Plate method.

Bacterial Isolates identified by API strips	Ampicillin (µg/mL)	Clotrimazole (µg/mL)	Novobiocin (µg/mL)	Tetracycline (µg/mL)
Pasteurella multocida	>128	32	64	8
Pseudomonas aeruginosa	64	> 128	32	4
Raoultella ornithinolytica	64	32	4	4
Tatumella ptyseos	32	> 128	4	4
Vibrio mimicus	64	8	8	8
Lactobacillus salivarius	8	8	4	8
Lactobacillus plantarum	32	64	64	2
Lactobacillus pentosus	64	> 128	64	2
Flavobacterium indologenes	>128	32	32	8
Sphingomonas multivorum	64	64	128	16
Pseudomonas fluorescens	128	64	64	8

From this list (**Table 3.6**), it appears that the selected bacterial isolates conferred robust resistance to both ampicillin and co-trimoxazole as indicated by MICs at >128µg/ml, which is an indication of uninterrupted growth. However, they exhibited variable resistance towards novobiocin and tetracycline. More importantly, five bacterial species (**Table 3.7**) showed resistance to all four antibiotics as demonstrated by MIC values greater than 128µg/ml. These five species were selected for further experimentation, including oxidase test and 16S rDNA

sequencing to confirm their taxonomic identification.

Table 3.7: Bacterial isolates exhibiting an MIC of ≥128 µg/mL for antibiotics Ampicillin, Clotrimazole, Novobiocin and Tetracycline, and were subsequently selected for further investigation.

Bacterial Isolates	Minimal Inhibitory Concentrations (Clinical and Laboratory Standards Institute., 2007) against selected antibiotics			
	Ampicillin (µg/mL)	Clotrimazole (µg/mL)	Novobiocin (µg/mL)	Tetracycline (µg/mL)
Burkholderia cepacia	>128	> 128	128	128
Pseudomonas stutzeri	>128	> 128	>128	>128
Enterobacter spp.	> 128	> 128	>128	>128
Enterobacter agglomerans	>128	> 128	>128	>128
Vibrio metschnikovii	>128	> 128	128	128

3.3.4 Oxidase test

All the selected bacterial isolates tested positive for oxidase, except for *Enterobacter agglomerans.*

3.3.5 Identification of bacteria using 16s rDNA analysis

The agarose gel containing the amplified 16S rRNA gene of the selected bacterial species is as shown in **Figure 3.4.** An expected amplicon size of ~370 was found in all the samples tested.

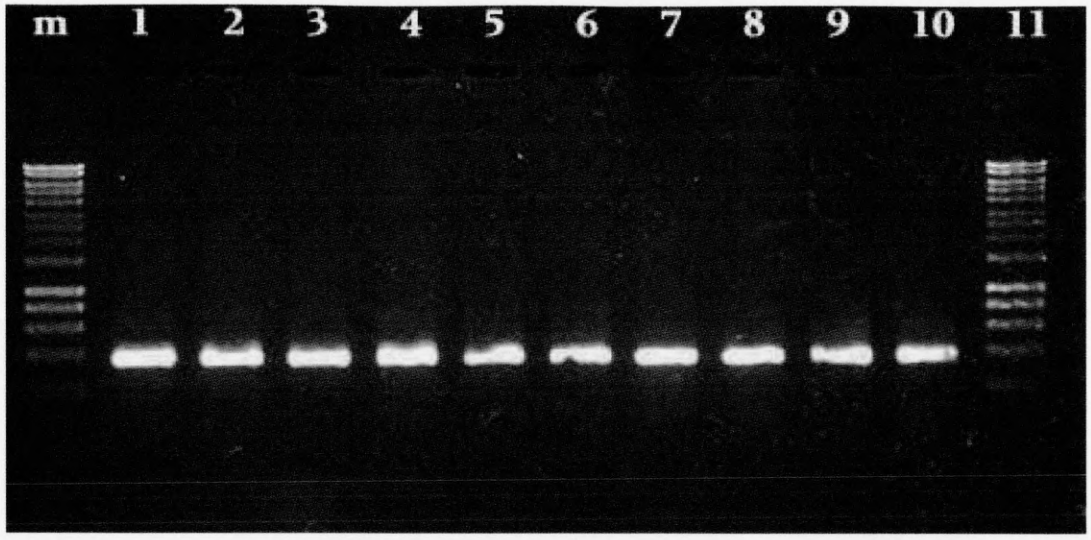

Figure 3.4: Agarose gel showing amplified 16S rRNA gene from selected bacterial species. Where; M & 11 = 1kb ladder; 1 = *Enterobacter* spp.; 2 = *Burkholderia cepacia*; 3 = *Aeromonas hydrophila*; 4 = *Enterobacter agglomerans;* 5 = *Pseudomonas stutzeri;* 6 = *Enterobacter* spp.; 7 = *Burkholderia cepacia*; 8 = *Aeromonas hydrophila*; 9 = *Enterobacter agglomerans;* 10 = *Pseudomonas stutzeri.* Lanes 1 to 5 using forward primer DG74 and reverse primer RW01 and lanes 6 to 10 using forward primer RDR080 and reverse primer PL06.

The amplicons were sequenced and analysed using the NCBI database (http://www.ncbi.nlm.nih.gov/, Accessed March 9, 2016) and BLAST software (http://www.ncbi.nlm.nih.gov/guide/howto/run-blast-local/, Accessed March 9, 2016) to reveal the identity of the test organisms. The result of BLAST analysis is as given in **Table 3.8**. The five strains were identified *Enterobacter spp.* (NCIMB14479), *Burkholderia cepacia* (NCIMB13694), *Aeromonas hydrophila* (NCIMB9239), *Enterobacter agglomerans* (NCIMB9680) and *Pseudomonas stutzeri* (NCIMB10783).

Table 3.8: Selected bacterial isolates identified by 16s rDNA gene sequencing method and subsequently selected for further experimental analysis.

Isolate 16s rDNA Identification	NCIMB Number
Enterobacter spp.	NCIMB14479
Burkholderia cepacia	NCIMB13694
Aeromonas hydrophila	NCIMB9239
Enterobacter agglomerans	NCIMB9680
Pseudomonas stutzeri	NCIMB10783

3.4 Discussion

The selection of the method used for the isolation of antibiotic-resistant strains from the river water is the single most important step in proving the hypothesis of this thesis. Among several methods available for water sampling, the membrane filtration (MF) technique was chosen in order to maximize the water sample volume, which in turn helps to maximize the number of bacteria isolated. The antibiotic sensitivity of the isolated bacteria was performed employing the classic disc agar diffusion technique. Results obtained from both antibiotic sensitivity assay using both M13 and M14 ring discs demonstrate the existence of multi-drug resistance in Gram-negative bacteria of this river environment. However, results also showed that different isolates display different sensitivities towards different antibiotics. For penicillin G, only 13 out of 146 strains were sensitive, whereas 133 exhibited resistance, which indicates that 91.09% of organisms tested can resist the antibiotic action of penicillin G, which makes this antibiotic, ineffective against many organisms. One possible reason can be that the penicillin G is the oldest known antibiotic and bacterial resistance has evolved since it was first developed. Hence, decreasing its effectiveness as an antibacterial agent. Ampicillin on the other hand, showed

some albeit less dramatic activity over that of penicillin G, displaying 21% efficacy. More importantly, bacterial isolates conferred significant resistance towards both β-lactam antibiotics, suggesting the presence of some unknown mechanisms that doesn't allow these compounds to enter the cell.

Penicillins consist of a large group of bicyclic ring compounds which contain a 4-membered β-lactam ring (called penams) fused to a 5-membered thiazolidine ring, which interferes with bacterial cell wall mucopeptide synthesis (Wise & Park, 1965). When penicillin-sensitive bacterial cells were exposed to penicillin, it resulted in the loss of cell wall integrity, leading to cell lysis and cell membrane damage beyond repair (Wise & Park, 1965). Benzylpenicillin (penicillin G) was the first natural penicillin with potent activity against all Gram-positive pathogens, Gram-negative cocci and some spirochaetes and actinomycetes (Nathwani & Wood, 1993). In the past 50 years, penicillin G has been the mainstay of therapy for serious pneumococcal, streptococcal, and gonococcal infections (Nathwani & Wood, 1993). However, the last decade has seen the emergence of bacterial resistance in certain parts of the world, initially among gonococci, and more recently among pneumococci and meningococci. The discovery of 6-aminopenicillinamic acid nucleus has led to considerable manipulation of the basic ring structure, resulting initially in the synthesis of ampicillin and subsequently, the other aminopenicillins, analogues, esters and prodrugs (Nathwani & Wood, 1993). These drugs have the advantage of improved oral bioavailability and superior activity against *Haemophilus influenzae* and certain Gram-negative bacilli including *Salmonella* spp. and *Listeria monocytogenes*, making these agents popular in the treatment of upper and lower respiratory tract infections and urinary tract infections(Nathwani & Wood, 1993). Cephalosporins were first introduced into chemical practice more than 30 years ago.

Cephalosporins, like penicillins, possess a β-lactam structure that is the critical determinant of their antibacterial activity (Harrison & Bratcher, 2008). The five-member thiazolidine ring, which is characteristic of penicillins, is replaced by a six-member dihydrothiazine ring in the cephalosporins (Prober, 1998). This ring is responsible for the compound's ability to resist inactivation by certain bacterial enzymes. Unlike the β-lactams, Cephalosporins are bactericidal. Following the penetration of the bacterial surface, they attach to the bacterial penicillin-binding proteins (PBPs), which catalyse reactions critical to cell wall synthesis and division (Prober, 1998). When the PBPs form complexes with cephalosporins, this catalytic activity is lost and cell wall synthesis and division are disrupted (Prober, 1998). The basic cephalosporin molecule has been modified extensively over the last three decades resulting in a proliferation of agents with diverse antibacterial, pharmacokinetic and pharmacodynamics properties (Nathwani & Wood, 1993; Harrison & Bratcher, 2008). Cephalosporins are grouped into *"generations"* based on the extent of their antimicrobial activity (Prober, 1998; Nathwani & Wood, 1993; Harrison & Bratcher, 2008). There are currently four generations of cephalosporins with numerous members per generation. Cephalothin, a first generation cephalosporin, was used in this study. Exposure of bacterial isolates to cephalothin showed 11% efficacy where it only inhibited 16 isolates leaving the rest (130 isolates) unaffected by the drug. Taken altogether, all three β-lactam compounds used in this experiment exerted comparable antimicrobial effect owing to the similarities in their chemical structure. Since all three compounds were structurally unaltered or slightly modified in the case of ampicillin, their antimicrobial activities were not high when compared to the extensively modified future generation drugs. In addition, a majority of the bacterial isolates showed high resistance to all three β-lactam compounds suggesting some unknown mechanisms that prevent bacterial cells

from taking up these drugs.

The tetracyclines are a family of antibiotics first discovered in the 1940s, which function to inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site resulting to the death of the organism (Chopra & Roberts, 2001). Tetracyclines are broad-spectrum antibiotics that target a wide range of Gram-positive and Gram-negative bacteria and other atypical organisms such as *Chlamydiae*, *Mycoplasmas*, *Rickettsiae* and protozoan parasites. The favourable antimicrobial properties of these agents and the absence of major adverse side effects have led to their extensive use in the therapy of human and animal infections (Chopra & Roberts, 2001). They are also used as prophylactic for the prevention of malaria caused by *Plasmodium falciparum*. In some countries, including the United States, tetracyclines are added at sub-therapeutic levels to animal feeds to act as growth promoters (Chopra & Roberts, 2001). Although tetracyclines retain important roles in both human and veterinary medicine, the emergence of microbial resistance has limited their effectiveness. In this study, exposure to tetracycline adversely inhibited the growth of 139 out of 146 bacterial isolates, which make tetracycline a potent antibiotic with 95% efficacy in these experiments.

Aminoglycosides are highly potent, broad-spectrum antibiotics with many desirable properties for the treatment of life-threatening infections. First developed in the 1940s, they are derived from antimicrobial substances produced by the soil-dwelling bacteria *Streptomyces* spp. and *Micromonospora* spp. (Avent *et al.*, 2011). The most widely known member of this group, gentamicin, has been used for the treatment of serious Gram-negative bacterial infections since the early 1960s. Aminoglycosides are bactericidal. Their primary site of action is the 30s subunit of the prokaryotic ribosome, interrupting bacterial protein synthesis. In

order to reach this active site, aminoglycosides bind to the bacterial cell wall and undergo active transport into the cytosol (Avent *et al.,* 2011). Streptomycin was the first aminoglycoside agent in widespread use, along with netilmicin and kanamycin, but kanamycin is now be infrequently used. With the advent of broad-spectrum β-lactam antimicrobials, such as anti- pseudomonal penicillins, third generation cephalosporins and carbapenems, there was a noticeable shift away from prolonged administration of aminoglycosides. This change has been driven by the improved safety profile and improved pharmacokinetic parameters of these newer drugs (Avent *et al.,* 2011).

In this experiment we used both streptomycin and gentamicin for the inhibition assay. There were duplicate experiments for streptomycin, since it was present in both M13 and M14 ring discs. Streptomycin exposure using the M13 ring disc showed 70.5% efficacy, where 103 isolates showed sensitivity and 43 were resistant. Similarly, streptomycin exposure, using the M14 ring disc showed 69% efficacy, where 101 isolates showed sensitivity, whereas 45 were resistant. These results affirm the reproducibility of both experiments. In addition, gentamicin displayed an improved antimicrobial efficacy of 85.6% compared to streptomycin, where it significantly inhibited the growth of 125 isolates, whereas 21 isolates were resistant to the drug. Despite the increase in drug efficacy, a small percentage of the isolates were able to confer resistance to these drugs, suggesting some unknown mechanisms that allow these organisms to exclude these substances or combat their bactericidal action.

Chloramphenicol is an antibiotic first isolated from cultures of *Streptomyces venezuelae* in 1947, but is now produced synthetically (Pongs, 1979). It was the first wide-spectrum antibiotic to be discovered and acts chiefly as a bacteriostatic agent (Pongs, 1979). It has a relatively simple structure and acts by interfering with bacterial protein synthesis more

specifically, it irreversibly binds to a receptor site on the 50s subunit of the bacterial ribosome inhibiting the enzyme peptidyltransferase (Pestka, 1974). This prevents amino acid transfer to the growing polypeptide chain and ultimately leading to inhibition of protein synthesis (Pestka, 1974). Due to its capacity to cause fatal aplastic anaemia in humans, chloramphenicol is prohibited in food and animals in the US and many other countries. In this experiment, exposure to chloramphenicol showed 40% efficacy, where 54 isolates showed sensitivity, while 92 were resistant to the drug. So far, this is the only drug among those that affects protein synthesis that produced the lowest level of sensitivity, suggesting that 92 of the bacterial isolates possess some unknown mechanisms prevent chloramphenicol from entering the cells.

Novobiocin is a member of the coumermycin family of antibiotics and is a well-established inhibitor of DNA gyrase (Burlison *et al.*, 2006; Luhrmann *et al.*, 1998). Novobiocin is produced by *Streptomyces* spp. (especially *S. niveaus* and *S. spheroides*) and its main targets are the Gram-positive bacteria. Novobiocin interferes with metabolic processes not only in bacteria, but also in eukaryotic cells. It has also been shown as a potent inhibitor of ADP ribosylation (Banasik *et al.*, 1992). In the present study, novobiocin exposure exhibited 12% efficacy, where only 18 isolates showed sensitivity, while the majority (128) of the isolates were able to resist the action of the drug.

The emergence of multidrug-resistant (MDR) Gram-negative bacteria in parallel with the lack of new antibacterial agents led scientists to discovery of polymyxins (Biswas *et al.*, 2012; Falagas & Kasiakou, 2005; Giamarellou & Poulakou, 2009). There has recently been a marked increase in infections caused by MDR-resistant strains of bacteria, including those caused by *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumonia*. For

these species, polymyxins are often available as active antibiotics (Falagas & Kasiakou, 2005; Jain & Danziger, 2004; Karabinis *et al.*, 2004; Michalopoulos *et al.*, 2005; Obritsch *et al.*, 2005). Polymyxins consist of polymyxins A-E, of which polymixin B (PMB) and polymixin E are currently available on the market. When the use of β-lactams, aminoglycosides or quinolones becomes ineffective, polymixins, especially colistin, serve as the final alternative treatment (Biswas *et al.*, 2012; Landman *et al.*, 2008). Colistin is an old class of cationic peptides, which act by disrupting bacterial membranes resulting in cellular death (Bialvaei & Samadi Kafil, 2015). Despite the daily selective pressure in patients receiving colistin by inhalation, resistance to colistin is seldom observed (Jensen *et al.*, 1987; Littlewood *et al.*, 2000). There are two forms of colistin available in the market, colistin sulfate and colistin methanesulfonate (colismethate sodium or CMS). Colistin methanesulfonate or CMS was largely replaced by the use of aminoglycosides in the 1970s, because of concern about its neurotoxicity and nephrotoxicity (Littlewood *et al.*, 2000). In the M14 sensitivity assay conducted in this project, 99 of the bacterial isolates showed inhibition of growth when exposed to colistin sulphate, while 47 were found to be resistant, suggesting that despite its efficacy some bacterial isolates can still obviate its bactericidal action of this drug by unknown mechanisms.

Pseudomonas stutzeri is a member of the genus *Pseudomonas*, that belongs to group I of Palleroni's DNA-rRNA homology group within the phylum Proteobacteria. *P. stutzeri* is now recognized as a member of class Gammaproteobacteria (Palleroni *et al.*, 1973; Palleroni, 2001). Phylogenetic studies of *P. stutzeri* strains' 16s rRNA sequences and other phylogenetic markers demonstrate that they belong to the same branch, together with related species within the genus, such as *P. mendocina, P. alcaligenes, P. pseudoalcaligenes and P. balearica*

(Lalucat *et al.*, 2006). Typically, *P. stutzeri* cells are rod shaped, about 1 to 3 μm in length and 0.5 μm in width, and have a single polar flagellum (Lalucat *et al.*, 2006). Phenotypic properties of the genus include a Gram-negative stain, positive catalase and positive oxidase tests, and a strictly oxidative metabolism. In addition, *P. stutzeri* strains are also defined as denitrifies (Lalucat *et al.*, 2006). They can grow on starch and maltose and have a negative reaction in arginine dihydrolase and glycogen hydrolysis tests. In addition, *P. stutzeri* does not produce fluorescent pigments, which differentiates it from other members of the fluorescent *Pseudomonas* spp. (Lalucat *et al.*, 2006).

Organisms of the *Burkholderia cepacia* complex (BCC) are Gram-negative bacteria of the β- proteobacteria subdivision that comprise at least 17 closely related species, which are genotypically distinct, but phenotypically similar. They include plant, animal and human pathogens, with a widespread distribution in natural and man-made habitats (Mahenthiralingam *et al.*, 2005). These bacteria exhibit an extraordinary metabolic versatility, which allows them to adapt to a wide range of environments. Among the BCC bacteria, several environmentally relevant strains have been identified due to their ability to degrade pollutants in water and soils (e.g., crude oils, herbicides, recalcitrant aromatic compounds and xenobiotics) (Sousa *et al.*, 2011). Several BCC strains are also able to produce antifungal compounds (Chiarini *et al.*, 2006) and some studies suggested that members of the *Burkholderia* genus are ancient nitrogen-fixing symbionts of Mimosa legumes, particularly adapted to acidic infertile soils (Bontemps *et al.*, 2010). Because of the ability of some strains to promote plant growth, BCC bacteria have attracted significant commercial interest as biocontrol, bioremediation and plant-growth promoting agents (Chiarini *et al.,* 2006). However, these bacteria have also emerged as important human pathogens and the risks

associated with the agricultural uses of BCC strains remain unclear. There is a general consensus that the large-scale use of organisms of the *Burkholderia* genus is imprudent until more is known about the fate of biocontrol strains after their release in the environment (Sousa *et al.,* 2011). For example, the epidemic *B. cenocepacia* strain PHDC was recovered both from patients suffering from cystic fibrosis (CF) in the mid-Atlantic region of USA, as well as from agricultural soils (LiPuma *et al.*, 2002). Aside from cystic fibrosis (CF), BCC bacteria are also important pathogens in compromised patients, such as patients suffering from chronic granulomatous disease (CGD) (Johnston, 2001), a rare hereditary disease caused by mutations in the subunits of the NADPH oxidase complex of the phagocytes, resulting in their inability to produce reactive oxygen species (Johnston, 2001). There are also some reports of BCC infections in immunocompromised patients of cancer and HIV and also among immunocompetent individuals (Mann *et al.*, 2010; Marioni *et al.*, 2006). In recent years, an increasing number of bacteraemia cases caused by BCC among non-CF hospitalized patients have been reported. Among these hospitalized non- CF patients; haemodialysis, permanence in intensive care units, use of central venous catheters, indwelling urinary catheters and endotracheal tubes are now recognized as risk factors contributing for BCC acquisition.

Strains from all the BCC species have been isolated from cystic fibrosis patients as well as from the environment. However, their frequency of isolation is uneven. While the majority of the isolates obtained belong to the species *B. cenocepacia* and *B. multivorans* (Mahenthiralingam *et al.*, 2005), the majority of the environmental isolates belong to the species *B. cepacia*, *B. ambifaria*, *B. cenocepacia* and *B. pyrrocinia* (Chiarini *et al.*, 2006).

One of the major problems associated with BCC infection is their intrinsic resistance to most of the clinically available antimicrobials, including aminoglycosides, quinolones,

polymyxins and β-lactams (Leitão *et al.*, 2008; Sousa *et al.*, 2011). The multi-resistance of BCC bacteria appears to result from various efflux pumps that efficiently remove antibiotics from the cell, decreased contact of antibiotics with the bacterial cell surface due to their ability to form biofilms and changes in the cell envelope that reduce the permeability of the membrane to the antibiotic (George *et al.*, 2009). BCC bacteria are also resistant to neutrophil-mediated non-oxidative killing and to the antimicrobial peptides produced by airway epithelial cells, including lysozyme, lactoferrin and phospholipase A2 (Baird *et al.*, 1999). Therefore, CF patients chronically infected with BCC are difficult to treat and, although current treatment strategies use double or triple antibiotic combinations to achieve bactericidal activity, they rarely result in the eradication of the pathogen, particularly in the case of chronic infection (George *et al.*, 2009).

Out of the 33 strains (Table 3.6) that were identified to the species level by API strips and further analysed using inhibition assays, five strains (Table 3.7) scored the highest resistance pattern to the four antibiotics with MICs >128μg/mL and were chosen for further study and identification, because they showed promising signs for multidrug resistance towards ampicillin and tetracycline Their rDNA sequences were compared against previously known 16s rDNA sequences to confirm their species identification.

16s rDNA gene sequencing is the currently the most preferred technique for bacterial identification. Due to widespread use of polymerase chain reaction (PCR) and DNA sequencing in the past decade, 16s rDNA (reverse transcribed from rRNA) sequencing has played a pivotal role in the accurate identification of unknown bacterial isolates and the discovery of novel bacteria in clinical microbiology and research laboratories (Woo *et al.*, 2008). 16s rDNA sequencing is particularly important for bacterial identification particularly in the case of bacteria with unusual phenotypic profiles, rare bacteria, slow-growing bacteria,

uncultivable bacteria and culture-negative infections. Not only has it provided insights into the aetiology of infectious disease, but it also helps clinicians and researchers in choosing antibiotics and in determining the duration of treatment and infection control procedures.

All five strains were processed for 16s rDNA sequencing followed by DNA sequence analysis. This is the final step in the identification process. Possible mechanisms for antibiotic resistance will be further studied through molecular studies of plasmid DNA as well as studies for protein expression.

3.5 Chapter Summary & Conclusions

Results obtained from the API strip identification system revealed that the Dighty Burn was contaminated with over 35 antibiotic-resistant bacterial strains belonging to the family *Enterobacteriaceae*, possibly brought to the river by runoff contamination from agricultural land surrounding the sampling site. These are enteric bacteria that normally reside inside the intestines of animals and humans. Moreover, both antibiotic sensitivity assays using the Mastring system and the 96-well plate system proved to be efficient methods for the determination of multidrug-resistance phenotypes of selected bacterial isolates. The API strip system provided a cheap, fast and efficient way to identify a large number of isolates compared to 16s rDNA sequencing. However, the latter provided a more specific taxonomic identification by comparing the sequences of rRNA gene to known sequences across the entire kingdom *Monera*. Results of API identification were all confirmed by 16s rDNA sequencing and we have now identified five novel multidrug-resistant species, which will be utilized for further characterization.

The next step in the experimentation process is to determine whether the multidrug resistance phenotypes displayed by these five species are encoded in the bacterial

chromosome or on a plasmid. After the determination of the origin of the multidrug resistance gene, subsequent experiments are required to investigate the presence of efflux pumps and their roles in multidrug resistance.

Chapter 4. Preliminary Analysis of Antibiotic Drug Resistance Mechanisms in Five Selected Gram-Negative Bacterial Species

This chapter addresses the experimental processes designed to identify the molecular mechanisms responsible for antibiotic resistance in the selected allochthonous bacterial strains isolated from the Dighty Burn. The Chapter includes an introduction (Section 4.1), the experimental approach (Section 4.2), results (Section 4.3), discussion (Section 4.4) and a chapter summary and conclusions (Section 4.5).

4.1 Introduction

The World Health Organization (WHO, 2015c) defines antimicrobial resistance as the ability of a microorganism to resist inhibition by an antimicrobial drug that was once able to treat an infection caused by that microorganism.

The evolution of resistance to antibiotics in bacteria is a powerful illustration of natural selection. As a result of clinical, prophylactic and agricultural overuse of antibiotics, the frequency of antibiotic resistance in certain bacterial populations has risen from undetectable levels to greater than 50% in less than 20 years (Watanabe 1963; Anderson 1968; Falkow *et al.*, 1971).

There are many molecular mechanisms by which the bacteria achieve this feat. The most important means through which bacteria gain resistance include:

- Plasmid mediated antibiotic resistance (Carattoli, 2009; Svara & Rankin, 2011)
- Efflux pump mediated drug resistance (Rouveix, 2007; Webber, 2003)
- Production of β-lactamase enzyme (Dever, 1991; Livermore & Brown, 2001).

Plasmid-mediated antibiotic resistance

An organism may exhibit resistance to one or multiple antibiotics as a dominant phenotype determined by the genes located on a plasmid. Plasmids are relatively small, circular DNA molecules, which replicate with some degree of autonomy in the bacterial cytoplasm. R-plasmids, which were initially called 'resistance factors' or R-factors, are small, extra-chromosomal genetic elements that play a central role in the dissemination and acquisition of resistance in bacteria. These resistant factors contain genes that facilitate the formation of sex pili, which are filamentous appendages on the cell surface (Petrocheilou *et al.,* 1976; Corpet & Lumeau 1987). This process consequently drives bacterial conjugation, which enables the transfer of a copy of a plasmid from the resistant organism to one which may have previously been drug-sensitive (Stone, 1975). The resulting ex-conjugants are then capable of acting as plasmid donors during subsequent pairings. Hence, R plasmids are commonly responsible for the epidemic spread of multiple drug resistance (MDR) throughout an entire bacterial population. This can pose serious problems in antibiotic therapy particularly as plasmids are often transmissible between organisms of different species and even different genera (Stone, 1975).

Sequencing of early generations of R plasmids demonstrated that most of their antibiotic resistant genes are often associated with transposable elements or transposons (Davies & Rownd 1972; Cohen *et al.,* 1972).Transposons are short sequences of DNA capable of changing location within the genome of a single cell. The majority of the well-characterized resistant plasmids isolated from Gram-negative bacteria are self-transmissible (conjugative) and carry more than one resistance gene. It is now believed that antibiotic resistance genes and plasmids existed even before the human use of antimicrobial drugs (Davies & Smith 1978; Hughes & Datta 1983). The phenomenon of plasmid-mediated transmissible drug resistance was first discovered in Japan in

late 1950s, following the isolation of dysentery causing strain of *Shigella flexneri* resistant to four drugs, namely chloramphenicol, tetracycline, streptomycin and sulphonamide (Kitamoto *et al.,* 1956). Multiple drug resistant *Shigella* strains were subsequently found with increasing frequency in clinical practice in Japan and became of particular interest, when it was observed that after administration of a single antibiotic, both sensitive and multi-drug resistant strains belonging to the same serological type could be isolated in specimens from either a single patient or multiple patients in the same outbreak (Meynell *et al.,* 1968). In addition, resistance also spread into other normal host flora present as commensals like *E. coli.* Researchers soon discovered that these bacteria acquired resistance through the spread of genes from one organism to another through horizontal gene transfer. The plasmids that mediated this transfer are called Resistance factors or R-factors (Normark & Normark 2002; Chandler *et al.,* 1977).

Organisms containing R plasmids carry genes that confer resistance to substances such as antibiotics (Ajamaluddin *et al.*, 2000), heavy metals (Nakahara *et al.*, 1977) or cellular toxins (Schmidt *et al.*, 1995). R plasmids, such as the R100, R1 and R6, all contain two functionally distinct units: (i) the Resistance transfer factor (RTF), which includes genes for plasmid replication and conjugation and (ii) the re-determinant (r-det) factor, which has the resistance genes and encodes for the production of enzymes that inactivate certain drugs or toxic substances (Legrand *et al.*, 1979). R-factors are usually assembled from multiple transposons. *Tn21* for instance, is a particularly remarkable example of a large composite transposon found in R100 plasmid, which contain mercury resistance genes in addition to genes for sulfonamide and aminoglycoside resistance (Liebert *et al.,* 1999). Different R-factors, when present in the same cell can recombine to produce R-factors with a new combination of genes in their re-determinants. Plasmids that carry genes to resist sulphonamides, streptomycins, chloramphenicol, tetracycline and mercury have been identified. This particular plasmid can be transferred between

a number of enteric species including *Escherichia, Klebsiella* and *Salmonella* (Brau & Piepersberg, 1983).

R-factors pose serious problems in treatment of infectious diseases with antibiotics. The transfer of resistance between bacterial cells of a population and even between the bacteria of different genera also contributes to the problem (Cohen *et al.*, 1972; Friesen *et al.*, 2006; Stone, 1975). Bacteria can conjugate and transfer plasmids and other genetic material to other cells within and outside their own species. For instance, it was previously found that resistance can be transferred widely among *Enterobacteriaceae*, *Vibrio cholerae*, *Pasteurella pestis* and *Serratia marcescens* (Farrar, 1985). It is thought that *Neisseria* spp. may have acquired its penicillinase producing plasmid from *Streptococcus*. and / or *Agrobacterium* spp. (Goh *et al.,* 1985). Specialized plasmid mediated transfer of DNA between certain bacterial species and plants cells have been characterized (Fründt *et al.,* 1998). Recombinant plasmids derived from cloning vectors are frequently lost from the host cells, even when they exist at high copy numbers. On the contrary, most natural R plasmids are remarkably stable and are rarely lost during multiplication of host cells, even when the copy number is low (Nordström & Austin, 1989). This is because R plasmids contain genes that ensure the correct partitioning of copies to daughter cells (Nordström & Austin, 1989). Moreover, some natural plasmids contain the *"killer elements"* composed of a stable killer protein or mRNA and an unstable inhibitor protein or antisense RNA, such that the loss of plasmids will result in the death of the host cells (Nordström & Austin, 1989).

Classic experiments by Avery and his group (1944) showed that *Pneumococcus* bacteria are *''transformed''* from normal to virulent when they take up DNA from virulent strains. However, natural transformation is a rare event, so Cohen and co-workers in 1973 (Cohen *et al.*, 1973) used a chemical method developed in 1970 by Mandel and Higa (1970). This involved mixing the bacteria and DNA in a suspension of cold $CaCl_2$ at freezing temperature. Then, Boyer

and Cohen rapidly raised and lowered the temperature to generate a *"heat shock"*. This technique induces the competence in bacteria to take up the plasmid DNA. These transformed bacteria were spread onto a culture plate containing the antibiotics tetracycline and kanamycin. Only transformed bacteria containing both kinds of resistance genes could grow in the presence of both antibiotics. This result was consistent with the bacteria being transformed with a recombined plasmid containing both the *tet* (tetracycline) and the *kan* (Kanamycin) resistance genes. However, it was also possible that some bacteria had been doubly transformed by re-ligated versions of the original plasmids. They can be easily differentiated from the transformed bacteria through restriction digestion of plasmid with subsequent visualization on agarose gel.

Antibiotic resistance prevents bacteria from antibiotic-induced damage and it can persist for long periods (Neu, 1992). The micro-ecological pressure exerted by the presence of an antibiotic is a potent stimulus to elicit a bacterial adaptation response and is the most common cause of bacterial resistance to antibiotics (Davies & Davies, 2010; Levin *et al.*, 2000; Normark & Normark, 2002). Antibiotic resistance could also be divided into (i) genetic drug resistance and (ii) phenotypic drug resistance. Genetic drug resistance is to the result of chromosomal mutations or the acquisition of antibiotic resistance genes on plasmids or transposons (Alanis, 2005).

4.1.1 Using bacterial transformation to identify the potential presence of antibiotic-resistance plasmids

Transformation involves the acquisition of genetic materials from the environment where it may be present in abundance, through cell death and disintegration, especially when an organism has been in competitive mode for survival (Boto, 2015; Lorenz & Wackernagel, 1994; Ochman *et al.*, 2000). There are certain conditions that need to be fulfilled for transformation to take place. Firstly, a competent cell capable of undergoing transformation must be available. Secondly, a

genetically active material must be provided and third, environmental conditions must be suitable for the union of two components of the system (Austrian, 1952). For transformation to occur, the bacterium requires high levels of available energy and should also be in a specific state of transformation-competence. Limitation of nutrients and high population density are some of the factors that lead to the state of competence required for this method of horizontal gene transfer (Thomas & Nielsen, 2005). In *Bacillus subtilis*, scarcity of amino acids at the end of exponential growth phase brings about the competence for transformation (Leisner *et al.,* 2008).

The intake of genetic material will only occur when compatible material, usually from the same species and suitable conditions are available (Nielsen, 1998). Although there are a few exceptions, this method cannot be a great driving force in widespread antibiotic resistance. In several species of *Streptococcus*, transformation takes place in high-density conditions (Li *et al.,* 2001) that result in the formation of bacterial biofilms that act as a reservoir of compatible genetic material.

Bacterial transformation systems can be divided into two categories, natural (or physiological) and artificial (Low & Porter, 1978). In naturally transformable species, the cells become competent (able to take up DNA from the environment) at a particular stage in the growth cycle. However, some species do not become naturally competent under normal culture conditions and must be rendered permeable to transforming DNA by artificial means. This may involve treating the whole cells with high concentrations of divalent cations such as Tris or Polyethylene glycol (PEG). Alternatively, all or part of the cell envelope exterior to the cytoplasmic membrane may be removed to form bacterial protoplasts or spheroplasts, which can then be induced to take up DNA by treatment with divalent cations or PEG.

In general, bacteria that can be transformed by chromosomal DNA can also be transformed by plasmid DNA. Transformation can be divided into three stages: First, is the

binding of the DNA to the cell surface receptors; second, involves the transport of DNA across the cell envelope; and third, is the establishment of the transforming DNA either as a replicon itself or by the combination with a resident replicon (Trautner & Spatz 1973; Weston *et al.,* 1981).

Two fundamentals elements have to be provided in order to perform bacterial transformation. The first element is an appropriate host bacterium. For this, *E. coli*™ DH5α™ can be used as host organism. Plasmid is the other vital component in the transformation technique. Plasmids usually encode for enzymes and antibiotic resistant markers that are expressed in the bacterium after transformation (Birnboim & Doly, 1979; Cohen *et al.*, 1973). The cells which have taken up the plasmid can be differentiated from cells, which have not taken up the plasmid by growing it on medium with ampicillin. Only the cells with the plasmid containing the ampicillin resistance (*amp*R) gene will survive. Furthermore, the transformed cells containing the plasmid with the gene of interest can be distinguished from cell with the plasmid, as well as those without an insert by the colour of the colony on media supplemented with IPTG and X-gal. Recombinants are white, whereas non- recombinants are blue in colour. The following is the diagrammatic representation of the pUC19 used in the present study **Figure 4.1**.

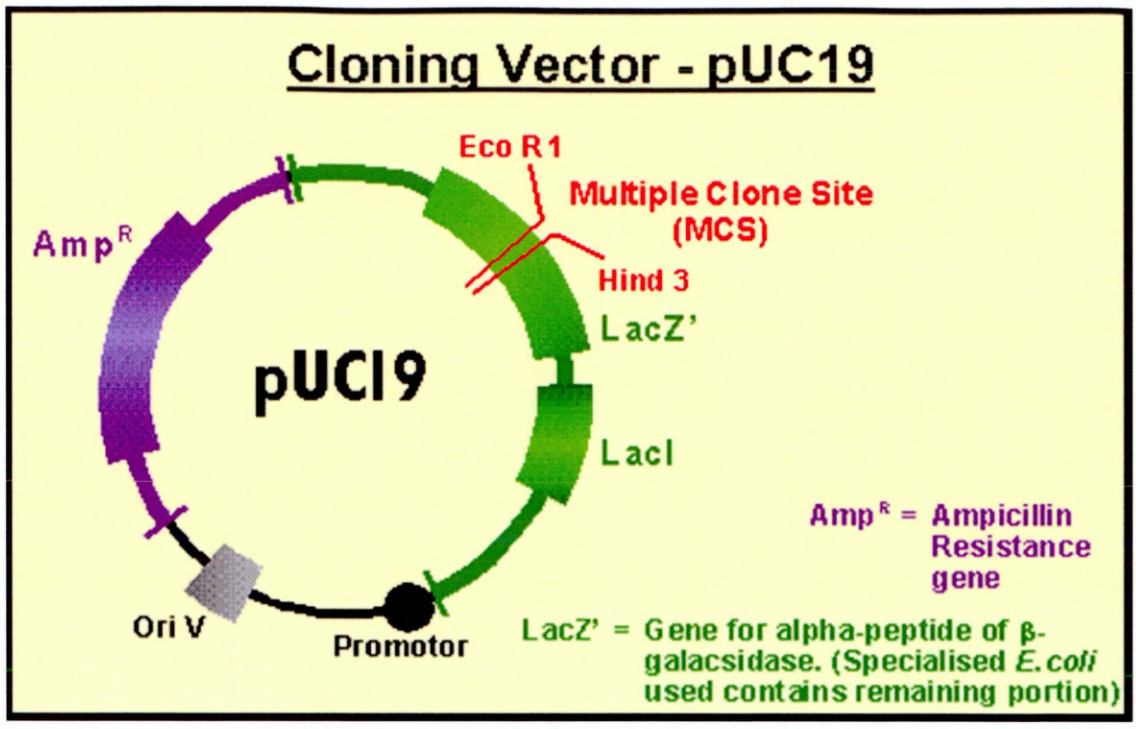

Figure 4.1: Map of the pUC19 plasmid. The pUC19 contains the ampicillin resistance gene (AmpR) (colour purple), the LacZ which has the multiple cloning sites (MCS) and the LacI genes. Absence of an insert at the MCS will result to the uninterrupted expression of the LacZ gene (blue colonies) while successful insertion at the MCS will disrupt LacZ gene expression resulting to white colonies. Adapted from Kaushik (2008).

4.1.2 Principal mechanism

DNA transformation is a naturally occurring phenomenon, by which DNA is transferred into a bacterial cell. However, under normal circumstances, this process rarely occurs in bacteria. The bacterial cell membrane is made up of lipid molecules that have negatively charged phosphate groups. During logarithmic growth, the cell membrane of *E. coli* contains hundreds of pores called *"adhesion zones"*. Even though adhesion zones are physically large enough to admit small loops of DNA, the negatively charged phosphate groups on the DNA helix are repelled by those on the lipids. Researchers use a combination of factors to make a bacterial cell capable of taking up new DNA. In 1970 Mandel and Higa discovered a way to make *E. coli* more *"competent"* in transforming foreign DNA by calcium chloride method and heat shock (Mandel & Higa, 1970).

When calcium chloride is added to the medium, it will interact with the negative charges of DNA helix of plasmid and the lipid membrane of bacterial cell to create an electrostatically neutral situation. Additionally, reduction in temperature aids in stabilising the negatively charged phosphates of lipid membrane such that calcium ions can easily shield them. A heat shock which creates a temperature imbalance of the bacterial membrane can then aid the extracellular DNA to pass through the adhesion zones.

4.1.3 Investigation of potential efflux pump mediated drug resistance

The term efflux pump is used to describe a family of transport proteins located on the cytoplasmic membrane of eukaryotic and prokaryotic cells (Wong *et al.,* 2014). They are also referred to as active transporters, because the transport function performed by these proteins is dependent on available chemical energy. Research has revealed that efflux pumps may be used specifically for the transport of a particular molecule or can transport various molecules with dissimilar structures (Fernández & Hancock, 2012). These efflux pumps are of two types, the primary active transporters, and secondary active transporters. Some of the active transporters use hydrolysis of adenosine triphosphate (ATP) as an energy source and are known as primary active transporters. Other types of pumps are known as secondary active transporters (also known as anti-porters/symporters/uniporters), where a difference in electrochemical potential across the cell membrane is created through pumping of ions, such as sodium or hydrogen (Fernández & Hancock, 2012).

There are five main super families of bacterial efflux transporters on the basis of the sequence of amino acids and the source of energy utilized for the export of substrates (**Figure 4.2**). They are:

- The Major Facilitator Superfamily (MFS) (Marger & Saier, 1993; Saidijam *et al.*, 2006)
- The ATP-Binding Cassette superfamily (ABC) (Konings & Poelarends, 2002; Méndez & Salas, 2001)
- The Small Multidrug Resistance family (SMR) (Chung & Saier, 2001)
- The Resistance-Nodulation-cell Division superfamily (RND)(Daniels & Ramos, 2009; McKeegan *et al.*, 2003)
- The Multi Antimicrobial Extrusion protein family (MATE) (Kuroda & Tsuchiya, 2009).

Only the ABC superfamily are primary transporters (utilising ATP), whereas the other four families are secondary transporters utilising proton differentials or sodium ion gradients as their energy sources (Sun *et al.,* 2014). In Gram-positive bacteria, most of the efflux transporters are MFS transporters, while the Gram-negative bacteria are mostly the RND family. Previously, it was thought that the RND family transporters were confined to Gram-negative bacteria, however they have now been shown to be located in every major kingdom of life (Morita *et al.,* 2006; Pao *et al.,* 1998; Lee *et al.,* 2001).

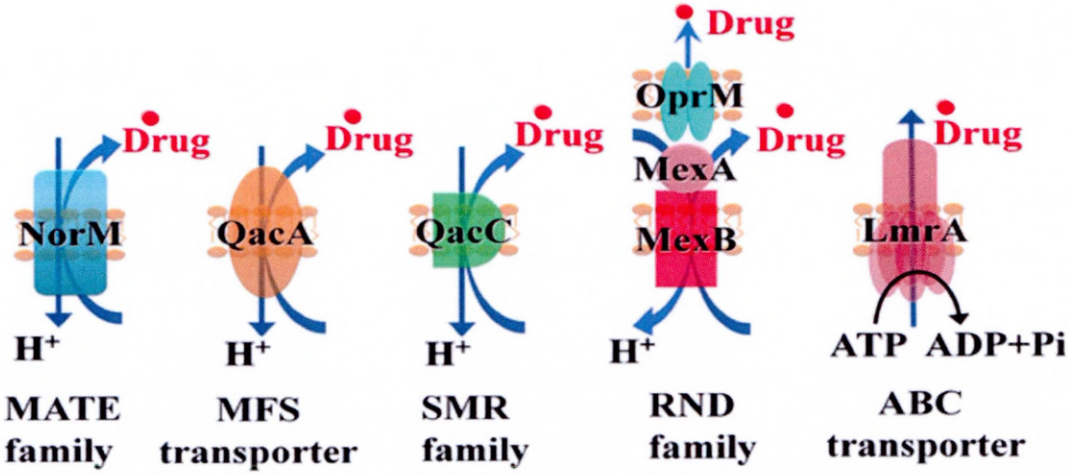

Figure 4.2: Schematic diagram showing of the five fundamental super-families of bacterial efflux systems: NorM, multi-antimicrobial extrusion protein family (MATE); QacA, major facilitator superfamily (MFS); QacC, small multidrug resistance family (SMR); MexAB, resistance-nodulation cell division superfamily (RND); LmrA, ATP-binding cassette superfamily (ABC). Adapted from Kourtesi *et al.* (2013).

4.1.4 Efflux pump mediated mechanism of antimicrobial resistance

Nikaido and Pagès (2012) indicated in their study that in the case of multidrug resistant *Pseudomonas aeruginosa*, the efflux pump plays a significant role in conferring drug resistance. There are four types of well-characterized efflux pumps systems in *Pseudomonas aeruginosa.* They are (i) MexE-MexF-OprN, (ii) MexX-MexY-OprM, (iii) MexC-MexD-OprJ and (iv) MexA-MexB-OprM (Sadeghifard *et al.,* 2012). The substrate specificities of these four pumps are different and the production of these pumps along with their activities can be heightened due to various factors that are usually present in case of infections (for example high bacterial inocula, stationary phase of growth, low pH etc.). Furthermore, constitutively Mex-Opr efflux pump system producing mutants are usually selected by fluoroquinolone antibiotics. Recent studies suggest that the higher prevalence of the efflux pumps is in the range of 14 to 75% in clinical strains of *Pseudomonas aeruginosa* (Aendekerk *et al.*, 2002; Aeschlimann, 2003; Evans

et al., 2001; Hocquet *et al.*, 2003; Kohler *et al.*, 1997; Li & Nikaido, 2009; Sugawara & Nikaido, 2014).

Some multidrug efflux pumps have the ability to expel a vast array of molecules from the periplasm or cytoplasm of bacterial cells, e.g. organic solvents, secondary metabolites and almost all clinically used antibiotics. Therefore, the significance of efflux mechanisms for providing antibiotic resistance to bacteria cannot be underestimated. Efflux, at a basic level is responsible for conferring intrinsic antibiotic resistance against specific antimicrobial agents. The observed resistance against antimicrobial drugs or agents is indicated by an observed increase in minimum inhibitory concentration (MIC). The constitutive increase of the efflux-pump protein expression is the major factor for increased MIC (Da Silva *et al.,* 2011). There are some clear instances which indicate that the efflux induced increase in MIC are enough to provide antibiotic resistance to the bacteria against the drug's breakpoint concentration (the concentration of antibiotic above which any bacterium in considered to be resistant) recommended to treat the infection. Fluoroquinolones most often exhibit this type of phenomenon (Piddock 2006; Fernando *et al.,* 2013).

The efflux pumps that confer antibiotic resistance to Gram-positive bacteria mainly belong to the MFS family, whereas those in Gram-negative bacteria mainly belong to the RND family (Mahamoud *et al.,* 2007). *E. coli* AcrAB–TolC drug substrate profile (RND family) includes various substances ranging from antibiotics, such as β-lactam antibiotics that are lipophilic, chloramphenicol, rifampin, tetracycline, nalidixic acid, fluoroquinolones, novobiocin and fusidic acid to other substrates like SDS, ethidium bromide, acriflavine, Triton X-100, short-chain fatty acids, bile salts and triclosan 27- 30.

Gastroenteritis is typically caused by *Salmonella typhimurium* and *S. enteritidis* (*Salmonella enteric* serovar *enteritidis*). 1 to 4% of all infections in humans are invasive

salmonellosis. Fluoroquinolones (e.g.: ciprofloxacin) or cephalosporin ceftriaxone are the most commonly administered antibiotics for treatment of this disease. In *S. typhimurium,* the AcrAB–TolC system functionally resembles the AcrAB–TolC system present in *E. coli* and its substrates are antibiotics such as quinolones, tetracycline, and chloramphenicol, as well as other compounds like acriflavine, ethidium bromide, Triton X-100, SDS and triclosan 38- 40 (Piddock, 2006). Many researchers have demonstrated that in the case of laboratory generated *S. typhimurium* mutants, along with human and veterinary *S. typhimurium* isolates, over expression of AcrB is related to MDR. The MICs of tetracycline, nalidixic acid and chloramphenicol for such strains were well above the recommended range (Kumar *et al.,* 2013; Du *et al.,* 2014; Ge *et al.,* 2009; Bavro *et al.,* 2008; Tikhonova & Zgurskaya 2004).

Clinically relevant Gram-positive bacteria like *Streptococcus pneumoniae* and *Staphylococcus aureus* express MDR efflux pumps (Piddock, 2006). Generally, soft-tissue and skin infections are caused by *S. aureus* and *S. pneumoniae* is responsible for meningitis, bronchitis and pneumonia. These types of infections are often fatal in both elderly and children (Appelbaum, 2002; Pintado *et al.*, 2012; Wardenburg *et al.*, 2007). The MFS efflux pump of *S. pneumoniae* usually exports antibiotics like ciprofloxacin, norfloxacin and fluoroquinolones along with the dyes such as ethidium bromide and acriflavine (Poole, 2000). The occurrence of *pmrA* over-expression in *S. pneumoniae* clinical isolates from various geographically discrete areas, have been determined by several research groups (Piddock, 2006). They located that the over-expression of *pmrA* is not only associated exclusively with *S. pneumoniae* isolates that are norfloxacin resistant, but also seen in the norfloxacin susceptible isolates. Recent studies have further noted the association of ABC-superfamily transporter over expression with resistance against ciprofloxacin, although their occurrence in clinical isolates is not yet confirmed (Garvey *et al.*, 2011). Additionally, the MFS efflux pumps Mef and the ABC superfamily transporter *Mel*

of *S. pneumoniae* confer macrolide resistance. In the pneumococcal genome, the location of the *mel* and *mef* genes is on the conjugative transposons related element suggesting that although initially they are not linked to the location of the chromosome, but later on these genes are attained and subsequently passed to the daughter cells (Piddock, 2006).

4.1.5 Tetracycline and Ampicillin resistance in different bacterial species

Tetracycline and ampicillin are two important antibiotics, which are widely used for clinical purposes. However, most of the Gram-positive and Gram-negative bacteria have the ability to develop resistance against these antibiotics. There are different mechanisms that are involved in the microbial resistance; however, the most clinically important mechanism is efflux pump mediated (Gottesman, 2002). The efflux pumps that confer ampicillin or tetracycline resistance to Gram-positive bacteria belong to MFS family of efflux pumps, whereas those efflux causing resistance in Gram-negative bacteria belong to RND family.

4.1.6 Efflux pump systems in *Enterobacter* spp.

Enterobacter spp. are Gram-negative, rod shaped, motile organisms located in diverse environmental conditions. They can be acquired from both exogenous and endogenous sources. The genome of this organism is closely associated with that of *Klebsiella* spp. and it is technically challenging to distinguish one from another. The *Enterobacter* species were rarely considered to be pathogens prior to the initiation of widespread usage of antibiotics. These species have been found to be responsible for nosocomial infections since the 1970s. A large focus has been given to their intrinsic antibiotic resistance and their role in neonatal infection. *Enterobacter agglomerans* contain the following genes for efflux transporters: *acr*B, efrA, *sug*E, *mep*A, *nor*E, *efr*B that produce several efflux pump systems.

4.1.6.1 The multi-drug efflux pump, EmmdR

There are twelve predicted multidrug efflux pump transmembrane, EmmdR in *Enterobacter cloacae*. A physiological investigation of efflux activities of antimicrobial agent has illustrated the EmmdR to be an H^+ / drug antiporter. However, it is not an efflux pump driven by Na^+, suggesting that it may be responsible for conferring multidrug resistance. Quinolones are actively pumped out of *E. cloacae* cells by EmmdR and pose as a key mechanism to provide multidrug resistance in these bacteria. This pump is most significant among all other pumps because it has the ability to induce resistance many drugs especially against antibiotics (Gao *et al.,* 2012) . In virulence cases of *E. cloacae,* the AcrAB-TolC efflux pumps are involved. The association of RobA, RamA and SoxS (regulatory proteins) in the *acr*AB up-regulation and providing antimicrobial resistance was established in *E. cloacae* by cloning and sequencing of the genes encoding these proteins.

4.1.6.2 SugE multidrug efflux pump

This efflux pump of *E. cloacae* is similar in nature and belongs to the SMR transporter family. They provide resistance against different antiseptics that are structurally distinct (Doumith *et al.,* 2009). At the same time they actively help in Ethidium bromide extradition. Thus, SugE has been shown to be an efflux pump for multiple drugs and falls under secondary active transporter that is proton driven (H+ /drug antiporter). In six *Enterobacter* isolates (including two belonging to the genus *Pantoea* and two to the genus *Salmonella*) the *efrB* and *efrA* genes were decoded. The ABC multidrug efflux pump EfrAB was first described in *Enterococcus faecalis* and is involved in resistance to antimicrobial agents that are structurally unrelated, for instance

tetraphenylphosphonium chloride, doxycycline, norfloxacin, doxorubicin, acriflavine, 40,6-diamidino-2-phenylindole, ciprofloxacin and daunorubicin (Lee *et al.,* 2001). The *Enterobacter* species have the capability to develop resistance against these antibiotics. Paterson (2006) had revealed that the most important mechanism responsible for drug resistance in most of the *Enterobacter* spp. is the efflux mechanism. The efflux systems that are involved in the tetracycline and ampicillin resistance in this species are SugE multidrug efflux pump, the AcrAB-TolC efflux pump system, the multi-drug efflux pump, EmmdR etc. Among these entire efflux pump, the most significant is the multi-drug efflux pump, EmmdR and SugE. Multi-drug efflux pump possess ten transmembrane sections that are responsible for the resistance of many antibiotics including the classes under consideration. The suppression activity of the multi-drug efflux pump is based on the production of quinolones (Piddock, 2006). The development of the resistance in this species is conferred by the increased production of quinolones and membrane protein, both of which has the property to suppress the activity of antibiotics by developing resistance. The SugE multidrug efflux pump is proton driven efflux pump which generates the resistance against these two classes of antibiotics with the aid of ethidium bromide extradition (Lee *et al.,* 2001).

Aeromonas spp. are Gram-negative bacilli that thrives in diverse aquatic environments (Khor *et al.*, 2015). They are opportunistic pathogens that cause infections in humans if they come into contact via derived products or contaminated water (Illanchezian *et al.*, 2010). Clinical manifestations of *Aeromonas* infection include infections in soft tissues and skin, as well as various clinical syndromes in patients that are (Gold & Salit, 1993). Multidrug resistance (MDR) phenotypes were noted among *Aeromonas* spp., a number of which were reversed through the use of phenylalanine-arginine-β-naphthylamide (PAβN), an inhibitor of efflux pumps (Giraud *et al.*, 2004; Lomovskaya & Watkins, 2001). Regarding MDR efflux pumps, no functional data has

been identified in *Aeromonas* species. However, the system of the RND family are said to key role in conferring antibiotic resistance to different Gram-negative bacilli present in diverse environmental conditions and act as opportunistic pathogens (Hernould *et al.,* 2008; Giraud *et al.,* 2004; Goñi-Urriza *et al.,* 2002).

Until now ten systems belonging to RND family have been identified that include seven of the hydrophobic/amphiphilic efflux-1 family (Saier & Paulsen, 2001; Seshadri *et al.*, 2006). Among them, the efflux pump that is most closely linked with the major AcrB system of *Escherichia coli* is the efflux B of *Aeromonas hydrophila* or AheB (Hernould *et al.,* 2008). They are phylogenetically related to the AcrA efflux pump, the *E. coli* AcrB efflux pump (69%) or RND efflux pump and TtgC, which is the outer-membrane protein of *Pseudomonas entomophila* (58%). The *ahe*R gene displays sequence identity with *TetR* family transcriptional repressors (Hernould *et al.,* 2008).

There is less functional data available on the efflux mechanism of *Aeromonas hydrophila* due to the lack of research (Hernould *et al.,* 2008). The major efflux mechanisms identified in this species are MDR phenotypes, amphiphilic efflux-1 family, and *Aeromonas hydrophila* efflux B. There are ten systems belonging to RND family have been identified that include seven of the hydrophobic/amphiphilic efflux-1 family causing the resistance against ampicillin and tetracycline (Seshadri *et al.*, 2006; Srikumar *et al.*, 1998). The minimum inhibitory concentration of both of these classes of antibiotics is affected by the amphiphilic efflux-1 family and *Aeromonas hydrophila* efflux B mechanisms. AheABC efflux pump is an important efflux pump which acts on different substrates and has the ability to induce resistance against nine different classes of antibiotics (Hernould *et al.,* 2008). This is the mechanism, which is used by *Aeromonas* spp. for developing resistance against the clinically used antibiotics. There are different levels of expression for this mechanism and therefore, the intensity of this mechanism

also differs (Hiroshi Nikaido & Pagès, 2012). Amphiphilic efflux-1 family is also associated with the resistance mechanism of different antibiotics (including tetracycline and ampicillin). However, the over expression of both of these pumps may lead to toxicity and serious clinically adverse effects in humans and other species (Drusano & Craig, 1997).

4.1.7 Efflux mechanisms in *Pseudomonas stutzeri*

In a study by Martinez *et al.*, (2010) it was found that isolates of *P. stutzeri* that exhibits fluoroquinolone resistance was linked to a minimum of *par*C and *gyr*A topoisomerase gene mutations. Studies related to plasmid mediated resistance to quinolone in *P. stutzeri* did not show positive results for genes such as *qnr*S, *qnr*A, *qnr*B, *qnr*VC, *qep*A and *xIb-cr* (Poirel *et al.*, 2010).

Antimicrobial susceptibility testing with or without the involvement of an efflux pump inhibitor, such as phenylalanylarginyl-β-naphthylamide (PAβN) (20mg/L), was performed in order to investigate the role of efflux pumps in resistance in the selected isolates. The MICs of ofloxacin, nalidixic acid, ciprofloxacin, norfloxacin, pefloxacin, moxifloxacin, enrofloxacin and levofloxacin are significantly decreased by PAβN addition (Poirel *et al.,* 2010). Similar findings were noted with rifampicin, tetracycline, chloramphenicol a number of β-lactams and trimethoprim. This suggests the involvement of efflux pump overexpression. In this case, no effect was noted for the aminoglycosides, such as gentamicin and tobramycin. This may be attributed to the TbtABM efflux pump of *P. stutzeri* that has already been identified previously as the factor responsible for conferring resistance to nalidixic acid, sulfamethoxazole, chloramphenicol and n-hexane (Poirel *et al.,* 2010).

TBT resistance in *P. stutzeri* is associated with an operon, known as tbtABM (Jude *et al.,* 2004). This has been demonstrated by *E. coli* cloning and overall increase of TBT MIC level by 20 folds. TbtABM is similar to proton-dependent efflux pumps of the membrane fusion (TbtA

analogue), RND (that is similar to TbtB), and outer membrane (TbtM type) proteins. These proteins function collectively to eliminate substrates across membranes of the Gram-negative bacteria. TBT is removed from *P. stutzeri* 5MP1 by TbtABM. In fact, other efflux pumps of RND from *Pseudomonas* sp., (MexAB-OprM system in *P. aeruginosa*) were cloned in *E. coli.* There they have shown to accommodate a similar substrates range as they do in the original host (Jude *et al.,* 2004).

The efflux pump that results in antibiotic resistance in *Aeromonas hydrophila* is the TbtABM efflux pump. The tributyltin, tetracyclines, and ampicillin resistance in these bacteria is caused by a multidrug resistant efflux pump, TbtABM efflux (Li & Nikaido, 2009). This efflux pump belongs to RND transporter family and uses TBT as the substrate. It was reported that different replicated form of this bacterial species are resistant to ampicillin and tetracyclines and reason behind this resistance was associated with the presence of TbtABM operon. There is a remarkable resemblance between this efflux pump and the efflux pump found in the other species described above. This efflux system is also tolerant to organic solvents and has high degree of homology with other multidrug resistant efflux mechanisms. Another important point of consideration for this efflux pump is that, this pump is specific to *Aeromonas* spp. only and differential expression is reported for the different members of this species.

The initial research carried out to understand the mechanism of drug resistance has made it clear that many bacterial species uses efflux mechanisms for drug resistance. Efflux pumps were studied for different species and classes and results in the resistance of most of antibiotics, for instance, for tetracycline and ampicillin, it is the dominant method for resistance. There are different efflux mechanisms in *Burkholderia cepacia*, e.g. in case of *Burkholderia pseudomallei*, there are genomes that encode several efflux systems, but important one is RND pumps, AmrAB-OprA, BpeEF and BpeAB-OprB (Mima & Schweizer, 2010).

AmrAB-OprA: This efflux pump is associated with the development of multidrug resistance in the different species and the most important targets of this efflux pump are the aminoglycosides, the tetracyclines, and ampicillin. Furthermore, this pump also has the capability to develop macrolide resistance (Kumar *et al.,* 2008).

4.1.8 Extended spectrum β-lactamases (ESBLs)

Members of the family *Enterobacteriaceae* commonly express plasmid-encoded β-lactamases (e.g.: TEM-1, TEM-2 and SHV-1). They confer resistance to penicillins, but not to extended-spectrum cephalosporins (Bradford, 2001). ESBLs are a group of enzymes that break down antibiotics belonging to the penicillin and cephalosporin groups and render them ineffective. ESBLs have generally been defined as transmissible β-lactamases that can be inhibited by clavulanic acid, tazobactam or sulbactam, and which are encoded by genes that can be exchanged between bacteria (Paterson & Bonomo, 2005; Walsh, 2003). In typical circumstances, 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 (Shaikh *et al.*, 2015). Therefore, antibiotic options in the treatment of ESBL-producing organisms are extremely limited.

4.1.8.1 Types of ESBLs

4.1.8.1.1 Sulfhydryl variant (SHV)

The SHV family of β-lactamases appears to be derived from *Klebsiella* spp and is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species (Haanpera *et al.*, 2008).

4.1.8.1.2 Plasmid encoded Transposable element β-lactamases (TEM-1)

TEM-1 is capable of hydrolysing penicillins and first generation cephalosporins, but is unable to attack the oxyimino cephalosporin. It is responsible for the ampicillin and penicillin resistance observed in *H. influenzae* and *N. gonorrhoeae* (Shaikh *et al.*, 2015).

4.1.8.1.3 Oxacillinases (OXA)

The OXA-type β-lactamases are so named because of their oxacillin-hydrolysing abilities. These β-lactamases are characterized by hydrolysis rates for cloxacillin and oxacillin greater than 50% as that for benzyl penicillin. They predominantly occur in *P. aeruginosa*, but have been detected in many other Gram-negative bacteria (Naas & Nordmann, 1999).

4.1.8.1.4 Cephalosporinases

Cephalosporinases are among the most abundant β-lactamases on the basis of the number of organisms that produce these enzymes. These cephalosporinases, frequently called species-specific AmpC enzymes, are often found in most Enterobacteriaceae as chromosomal enzymes. AmpC β-lactamases, in contrast to ESBLs, hydrolyse broad and extended-spectrum cephalosporins (cephamycins as well as to oxyimino-β-lactams), but are not inhibited by β-lactamase inhibitors such as clavulanic acid (Bush, 2010a).

4.1.8.1.5 Studies into the role of potential β-lactamase involvement in observed antibiotic resistance

The integrity of the bacterial cell wall is essential to maintaining cell shape in a hypertonic and hostile environment (Massova & Mobashery, 1998). β-Lactam antibiotics exhibit their bactericidal effects by inhibiting enzymes involved in cell wall synthesis (Drawz & Bonomo,

2010). The β-lactam nucleus, 6-aminopenicillanic acid (6-APA) proved to be the key in penicillin synthesis and modification (Kong *et al.,* 2010). Among the β-lactam antibiotics of clinical utility for the treatment of infections caused by susceptible Gram-negative bacteria are penicillins such as amoxicillin, oral cephalosporins (such as cefpodoxime and cefuroxime and axetil), parenteral cephalosporins (such as cefepime and ceftriaxone) and the carbapenems (such as doripenem, ertapenem, imipenem and meropenem).

Unfortunately, the efficacy of these life-saving antibiotics is significantly threatened by bacterial 'β-lactamases' (Drawz & Bonomo, 2010). It is the most effective way for bacteria to counteract β-lactam antibiotics is by producing β-lactamases. These are enzymes that inactivate the drugs by hydrolysing the β-lactam ring (Kong *et al.,* 2010). Through hydrolysis, the lactamase enzyme breaks the β-lactam ring open, deactivating the molecule's antibacterial properties. Penicillinase was the first β-lactamase to be identified h (Queener, 1986). It was first isolated by Abraham and Chain in 1940 from Gram-negative *E. coli* even before penicillin entered in the clinical use. These enzymes have the ability to hydrolyse the β-lactam chemical bond that distinguishes β-lactam antibiotics from other antibacterial agents, thereby rendering the molecules incapable of killing bacteria. Today, over 890 unique β-lactamases have been identified in naturally occurring bacterial isolates (Bush & Jacoby, 2010 & 2015) β-lactamases are responsible for resistance to penicillins, extended-spectrum cephalosporins, monobactams, and carbapenems (Bush, 2010).

4.2 Experimental Approach

It is important to distinguish the several ways in which a microorganism may demonstrate resistance. This chapter therefore will investigate and elaborate the bacteria resistance mechanism. Resistant pathogens use several strategies to avoid the effects of antimicrobial

agents, biochemical, or genetic aspects and have evolved highly efficient means for clonal spread and for the dissemination of resistance traits. The major objective of this chapter is to experimentally analyse the underlying mechanism of antibiotic resistance in a group of preselected bacterial pathogens.

4.2.1 Bacterial culture

The five bacterial isolates were selected for the study based on their resistance towards specific antibiotics. *Pseudomonas stutzeri*, *Burkholderia cepacia*, *Aeromonas hydrophila, Enterobacter agglomerans* and *Enterobacter* spp. were the bacterial isolates used.

4.2.2 Screening for plasmid mediated antibiotic resistance

4.2.2.1 Plasmid extraction

Plasmid DNA extraction was carried out as previously described (Kado & Liu 1981; Sathyanarayana & Verma 1993) with some modifications. The methodology followed for plasmid extraction from bacteria is given in **Chapter 2** section **2.10.7.**

4.2.2.2 Bacterial transformation

E.coli DH5α was used as host organism for transformation and competent cell preparation was done using calcium chloride. Bacterial transformation was performed with 1μL of pUC19 vector (without insert) as positive control and plasmids isolated from test organisms using DH5α^{TM} competent *E. coli* cells. Bacterial transformation was done by the temperature heat shock method. The competent cell preparation and transformation experiment was conducted as per (Sambrook *et al.,* 1989). The procedure followed is described in **Chapter 2** sections **2.10.8** and **2.10.9**. The transformed cells were then plated onto ampicillin containing LB agar plates. LB-agar plates

were made with varying concentrations of ampicillin (to give final concentrations of 16, 32, 64 and 128 µg/mL), 100µl of the mixture from the transformation tubes was carefully transferred on to duplicate LB-agar plate and spread evenly with the help of a sterile glass spreader. The plates were then incubated overnight at 37°C to allow the transformed bacteria to form colonies.

4.2.3 Screening for efflux pump mediated antibiotic resistance

The efflux inhibitor, carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) was used is order to determine the role of efflux pumps in conferring antibiotic resistance in the five selected bacterial isolates. The five isolates were individually grown overnight in LB broth containing ampicillin and tetracycline with or without the CCCP and the resultant MIC was determined. The procedure followed for screening the presence of efflux pump is as described in **Chapter 2** section **2.10.10.**

4.2.4 Determination of penicillinase activity

Penicillinase activity and beta-lactamase production was the determined using the one minute tube test and the tube dilution test employing a wide range of ampicillin concentrations (0.5-128µg/mL), with and without the β-lactamase inhibitor, clavulanic acid (16µg/mL). The clavulanic acid concentration used was the lowest MIC breakpoint for the isolates. Two methods employed include phenol red indication method and tube dilution test. Refer **Chapter 2** section **2.11** for the detailed procedure followed for screening penicillinase production in test organisms.

4.3 Results

4.3.1 Plasmid mediated drug resistance

The results of the transformation experiment are as given in **Table: 4.1**. DH5α *E. coli* transformed with *Burkholderia cepacia* and *Aeromonas hydrophila* plasmids were able to grow in ampicillin containing LB agar plates indicating the role of plasmids in transfer of drug resistance. DH5α *E. coli* transformed with *Enterobacter agglomerans, Enterobacter* spp. and *Pseudomonas stutzeri* plasmids however failed to grow in ampicillin containing LB agar plates.

Table 4.1: Results of the transformation of plasmids from *Pseudomonas stutzeri*, *Burkholderia cepacia*, *Aeromonas hydrophila*, *Enterobacter agglomerans* and Enterobacter spp. via heat shock technique using DH5α *E. coli* as the competent cell. Growth and determination of MIC for transformed *E. coli* in the presence of varying concentrations of Ampicillin in LB-agar plates.

Bacterial isolates	Ampicillin concentration (µg/mL)					Growth after 48 hours
	MIC	16	32	64	128	
Enterobacter agglomerans	64	-	-	-	-	No growth
Burkholderia cepacia	128	+	+	+	+	Growth
Aeromonas hydrophila	128	+	+	+	-	Growth
Enterobacter spp.	128	-	-	-	-	No growth
Pseudomonas stutzeri	16	-	-	-	-	No growth
Negative control (Sterilized water)	-	-	-	-	-	No growth
Positive control (PUC19)	-	+	+	+	+	Growth

The results of the experiment exhibited the role of plasmids in mediating drug resistance in *Burkholderia cepacia* and *Aeromonas hydrophila*. However, the mechanism of drug resistance in

Enterobacter agglomerans, Enterobacter spp. and *Pseudomonas stutzeri* still remains to be identified.

Recent studies have suggested that efflux pumps play a prominent role in the multidrug resistance in many clinically important Gram-negative bacteria. Thus, the following study was undertaken in an attempt to determine the role of efflux pumps in multi-drug resistance in the selected group of Gram-negative organisms. The presence of more than one type of drug resistance mechanism is also possible; so all the bacterial group of the previous study was included.

4.3.2 Efflux pump mediated drug resistance

The result of the experiment is as given in **Table 4.2**. In *Enterobacter agglomerans* and *Enterobacter* spp. there was a 4 and 3 fold reduction in MIC of antibiotics respectively, strongly suggesting the role of efflux pumps in conferring drug resistance. In the case of *Burkholderia cepacia, Aeromonas hydrophila* and *P. stutzeri* there was no reduction in MIC suggesting the absence of an efflux pump mediated resistance mechanism.

Table 4.2: Comparative analysis of MIC of ampicillin and tetracycline with and without the efflux pump inhibitor, CCCP for *Pseudomonas stutzeri*, *Burkholderia cepacia*, *Aeromonas hydrophila*, *Enterobacter agglomerans* and *Enterobacter* spp. Where, Tet. = Tetracycline

Bacterial isolates	MIC values with or without efflux inhibitor CCCP				
	Ampicillin (µg/mL)	Tet. (µg/mL)	Ampicillin (+ CCCP) (µg/mL)	Tet. (+ CCCP) (µg/mL)	Fold reduction of MIC
Enterobacter agglomerans	64	32	4	4	4
Burkholderia cepacia	128	128	128	128	0
Aeromonas hydrophila	128	0.5	128	0.5	0
Enterobacter spp.	128	0.5	8	1	3
Pseudomonas stutzeri	16	0.5	16	0.5	0

The previous two studies focussing on role of plasmids and efflux pumps in mediating antibiotic resistance revealed that plasmid mediate drug resistance in *Burkholderia cepacia* and *Aeromonas hydrophila* and efflux pump mediate drug resistance in *Enterobacter agglomerans* and *Enterobacter* spp. *Pseudomonas stutzeri* was found to harbour neither of the above said mechanisms. Production of β-lactamase enzymes that degrade β-lactam antibiotics is reported to be one of the most widespread mechanisms of antibiotic resistance. Thus, the following study focussed on determining the presence of β-lactamase activity in the selected group of antibiotic resistant isolates. The presence of more than one type of drug resistance mechanism is also possible, so all the bacterial isolates from the previous studies were included in this study.

4.3.3 β-lactamase mediated drug resistance

The results of the MIC tube dilution method for screening the presence of β-lactamases are given in **Table 4.3**. No positive correlation between the presence of clavulanic acid and the MIC was observed in this study. In the case of *B. cepacia*, *Aeromonas hydrophila* and *Enterobacter* spp. the MIC remained the same (i.e. 128 μg/mL) suggesting the absence of β-lactamase enzymes. In the case of *Enterobacter agglomerans* and *P. stutzeri* there was an increase in MIC suggestive of a negative result.

Table 4.3: Comparative analysis of MIC of Ampicillin in presence and absence of Clavulanic acid (16 μg/mL) for *Pseudomonas stutzeri*, *Burkholderia cepacia*, *Aeromonas hydrophila*, *Enterobacter agglomerans* and *Enterobacter* spp.

Bacterial isolates	MIC of Ampicillin (μg/mL)	MIC of Ampicillin + 16μg/mL Clavulanic acid (μg/mL)
Enterobacter agglomerans	64	128
Burkholderia cepacia	128	128
Aeromonas hydrophila	128	128
Enterobacter spp.	128	128
Pseudomonas stutzeri	16	32

4.4 Discussion

The role of plasmids in antibiotic resistance in environmental bacterial isolates has been proven through this study. This study also describes the development and use of a novel method for the isolation of plasmids. The original protocol developed for plasmid extraction from conventional bacterial models, like DH5α may not necessarily yield good results with other bacterial species.

Hence, major changes in incubation period and centrifugation speed/time were incorporated. In conventional methods after the treatment with cell lysis buffer the incubation period is only 5 minutes. In the present study it was prolonged to one hour, which provides better results in terms of isolating plasmids from bacteria under study. Furthermore, the centrifugation speed and time were also increased, which in turn resulted in better yield bacterial plasmids.

The pUC19 vector containing the ampicillin resistance genes from *Burkholderia cepacia* and *Aeromonas hydrophila* made the DH5α cells resistant towards ampicillin as evidenced by its growth in ampicillin containing agar plates. This strongly indicates the role of plasmid in transferring drug resistance. The failure for DH5α containing other bacterial plasmids to grow in presence of antibiotic can be attributed to several factors. First, the transformation may not have occurred, as not all types of bacterial cells are capable of engaging in the transformation processes. Secondly, it is possible that the bacterial isolates tested carry antibiotic resistance via other mechanisms and not via a plasmid. Finally, sometimes bacteria lose their ability to maintain the plasmid in the newly transformed cells (Al-Allaf *et al.*, 2013). In the case of the transformed DH5α containing *B. cepacia* plasmid growth was observed in 128μg/μL ampicillin. In case of DH5α containing plasmid from *Aeromonas hydrophila,* the growth was inhibited at 128μg/mL concentration of ampicillin, which implies that addition of plasmid does not improve the resistance of this organism to the antibiotic ampicillin.

The failure of *E. agglomerans*, *Enterobacter* spp. and *Pseudomonas stutzeri* to transform suggests a role of other molecular mechanisms conferring resistance, e.g. efflux pump mediated drug resistance. Therefore, the next experiment was planned and executed to study more about the role of efflux pumps in conferring drug resistance. Drug efflux is a key multi-drug resistance mechanism reported among Gram-negative bacteria. Efflux pumps allow the microorganisms to regulate their internal environment by removing toxic substances, including antimicrobial agents.

These systems actively pump antibiotics out of the bacterial cell, hence maintaining a sub-MIC internal level of antibiotic and conferring protection against the antibiotic therapy. The role of efflux pumps in drug resistance is thus proven in this study.

The presence of CCCP in the medium resulted in a reduction of minimum inhibitory concentration from 64µg/mL to only 4µg/mL in the case of *E. agglomerans* bacteria. This indicates a correlation between the inhibition of the efflux pumps and resistance to antibiotics. It is thus proven that efflux pumps have a significant role in conferring resistance to *E. agglomerans* against ampicillin. *Enterobacter* spp. exhibited a 3-fold decrease in MIC on the addition of an efflux pump inhibitor. Therefore, we can conclude that there is a significant role of efflux pumps in the resistance of both the *Enterobacter* species.

In the case of *B. cepacia* no change in resistance was found when the inhibitor was added. This might be due to the presence of a different antibiotic resistant mechanism in these bacteria. The resistance of *A. hydrophila* and *P. stutzeri* was also found to be unaffected by the addition of CCCP. Both of these microorganisms were found to be highly susceptible to tetracycline, as compared to ampicillin, with an MIC of only 0.5µg/mL. It is clear that the efflux pump has no role in the resistance mechanism of these organisms.

With the above two studies, the role of plasmid mediated and efflux mediated drug resistance have been proven. However, *B. cepacia* was found to have none of the above stated drug resistant systems. Another important method by which bacterial pathogen achieves multi-drug resistance is via production of β-lactamase enzyme. β-lactams are the most commonly used antibiotics and therefore, β-lactamases are the most important source of resistance to them. Therefore, the next set of experiments was focused on understanding more about the β-lactamase enzyme and its subsequent identification in the bacterial groups under study.

The potential role of β-lactamases in conferring the observed antibacterial resistance was screened using clavulanic acid, a broad-spectrum inhibitor of enterobacterial β-lactamases. β-lactam antibiotics have been studied in combination with clavulanic acid. Three bacterial stains, *B. cepacia, A. hydrophila* and *Enterobacter* spp. exhibit minimum inhibitory concentrations (MIC) of 128μg/mL when ampicillin was used alone. However, no change was observed in MIC, when clavulanic acid was used in combination. This indicates that these organisms do not produce β-lactamases enzyme and therefore, β-lactamases inhibitors have effect in conferring anti-bacterial resistance.

The lower MIC value observed for *Enterobacter agglomerans* and *P. stutzeri* than indicates their susceptibility against ampicillin. An increase in the MIC by 2 fold, i.e. 128 and 32, in the presence of clavulanic acid is interpreted as a negative result. The presence of a sub-inhibitory level of clavulanic acid (8μg/mL) may sometimes lower the minimum inhibitory concentration of ampicillin for many resistant *Enterobacteriaceae* (Finlay *et al.,* 2003). The large differences among various bacterial species and different strains of the same species in their response to clavulanic acid cannot be predicted. The effect of the β-lactamase inhibitor also depends on the type and amount of β-lactamase produced (if formed), the location of the lactamase (intra- or extracellular), the penetration of the β-lactam antibiotic into the cell, the stability of the -lactam antibiotic to β-lactamases, and the activity of the β-lactam antibiotic used.

4.5 Chapter Summary & Conclusions

Thus, it can be concluded that plasmid and efflux pump mediated drug resistance mechanisms have an important role in conferring resistance to various Gram-negative microorganisms under study. The resistance in *Burkholderia cepacia* and *Aeromonas hydrophila* was found to be plasmid mediated and in the case of *Enterobacter agglomerans* and *Enterobacter* spp. it was

efflux pump mediated. The antibiotic resistance mechanism of *Pseudomonas stutzeri* still remains to be elucidated.

The next logical step was to identify and characterize the major protein families, which play an important role in making bacterial pathogens resistant towards antibiotics. The main methodology adopted includes extraction and purification of bacterial proteins, molecular characterisation of purified proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and genomic characterisation of bacterial DNA by polymerase chain reaction (PCR).

Chapter 5. Proteomic and Genomic Characterization of Five Isolated and Selected Bacterial Species

This chapter describes the experimental processes designed for proteomic and genomic characterization of the selected allochthonous bacterial strains isolated from the Dighty Burn. The Chapter includes an introduction (Section 5.1), the experimental approach (Section 5.2), results (Section 5.3), discussion (Section 5.4) and a chapter conclusions and summary (Section 5.5).

5.1 Introduction

Hosts and their symbiont bacteria have coevolved for many years to achieve their present status of mutually beneficial co-existence. During this long period the bacteria have updated their virulence mechanisms in order to counter the host defence systems and therefore, they have become generally more pathogenic (Beceiro *et al.,* 2013). The efficiency of various bacterial pathogens to infect and cause disease in their corresponding host often depends upon a number of virulence factors acting individually or together at different stages of infection. Virulence factors of pathogenic bacteria aid in successful interactions with the host tissues by effectively masking the bacterial surface from the host's defence mechanisms (Wu *et al.,* 2008). Thus, identifying the virulence factors is of paramount importance in understanding the bacterial pathogenesis and their interactions with the host. This knowledge will help in devising new methods to counter act bacterial infections (Wu *et al.,* 2008).

The cell envelope of bacteria is a complex multi-layered structure that serves as a protective barrier to ensure the organism's survival in diverse unfavourable environments (Silhavy *et al.,* 2010; Martorana *et al.,* 2014). Bacterial cell envelopes fall into two major

categories: Gram-negative bacteria are surrounded by a thin peptidoglycan cell wall, which itself is surrounded by an outer membrane (OM) containing lipopolysaccharide (LPS) (Silhavy *et al.,* 2010). On the other hand, Gram-positive bacteria lack an outer membrane (OM), but are surrounded by layers of peptidoglycan many times thicker than those found in Gram-negative bacteria (Silhavy *et al.,* 2010). Bacterial membranes and other structures that protect and encapsulate the cytoplasm do not resemble a simple membrane. Unlike the cells of eukaryotic organisms, the bacterium is constantly subjected to unpredictable, dilute and often unfavourable environments (Silhavy *et al.,* 2010). In order to survive, bacteria have evolved a sophisticated and complex cell envelope that not only acts as a protective barrier, but also allows selective transport of nutrients from the outside of the cell and waste products from the inside of the cell (Silhavy *et al.,* 2010). There are three principal layers of the bacterial cell envelope: the outer membrane (OM, in Gram-negative cells only), the peptidoglycan cell wall and the cytoplasmic or inner membrane. The two membrane layers confine the aqueous cellular components of the Gram-negative bacterial cell (Silhavy *et al.,* 2010) **(Figure 5.1).**

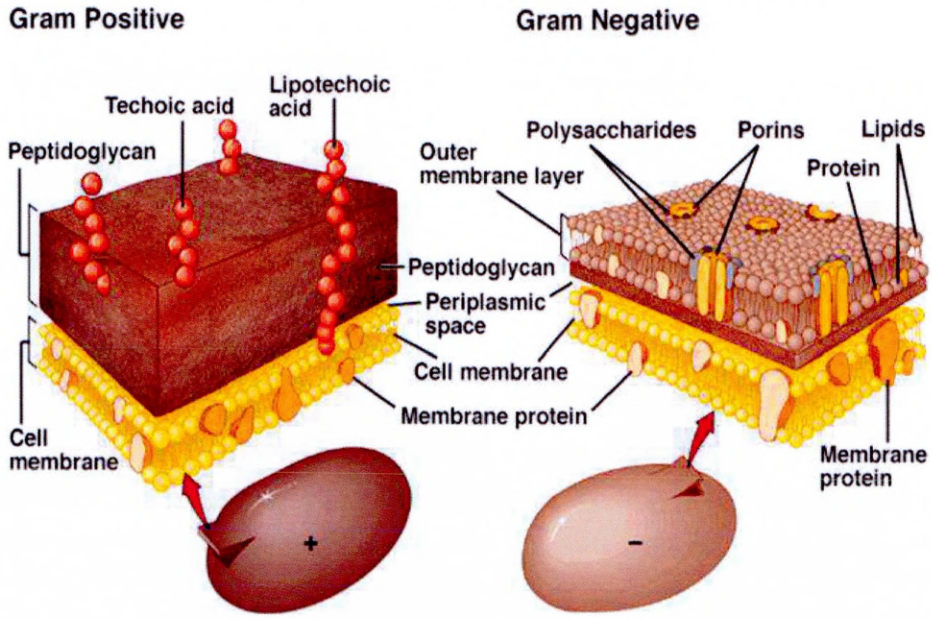

Figure 5.1: Schematic diagram showing Gram-positive (left) and Gram-negative (right) bacterial cell wall structures. Gram-positive bacteria are surrounded by a thick peptidoglycan layer and Gram-negative bacteria are surrounded by a thin peptidoglycan cell wall and outer membrane (OM, containing lipopolysaccharide (LPS). (http://medimoon.com/2013/04/why-is-it-more-difficult-to-treat-gram-negative-bacteria/) (Accessed on 20/11/2015) (Hayat, 2013).

5.1.1 The Outer membrane (OM)

The outermost layer that surrounds most Gram-negative bacteria is the outer membrane (OM), which separates the bacterial periplasm from the extracellular milieu. This organelle is the distinguishing feature of Gram-negative bacteria, because Gram-positive bacteria do not possess this organelle (Silhavy *et al.,* 2010). The OM in Gram-negative bacteria is a unique lipid bilayer. It is asymmetric in nature; where phospholipids are restricted only to the inner leaflet of the OM, while the outer leaflet is composed of glycolipids mostly, lipopolysaccharide (LPS) (Nakamura *et al.,* 1987; Raetz & Whitfield 2002; Wu *et al.,* 2014). Embedded in the OM are outer membrane proteins (OMPs), such as lipoproteins and β-barrel proteins that perform activities crucial to OM function. Based on its structural composition, the OM provides an effective barrier that is crucial

for the survival of organisms in many distinct environments and also renders Gram-negative bacteria more resistant to antibiotics than their Gram-positive counterparts (Wu *et al.,* 2014).

5.1.2 Outer membrane proteins

Proteins embedded within the OM are divided into two classes: lipoproteins and β-barrel proteins (Sankaran & Wu, 1994). Bacterial lipoproteins are a set of membrane proteins with many distinct functions. They have been shown to perform various roles including nutrient uptake, signal transduction, adhesion, conjugation and sporulation (Alloing *et al.*, 1994; Lampen & Nielsen, 1984; Mathiopoulos *et al.*, 1991; Perego *et al.*, 1991; Sutcliffe & Russell, 1995) and participate in antibiotic resistance transport (such as ABC transporter system) and extra-cytoplasmic folding of proteins (Kovacs-Simon *et al.,* 2011; Alloing *et al.,* 1994; Hussain & Lampen 1983; Mathiopoulos *et al.,* 1991; Sutcliffe & Russell 1995). In the case of pathogens, lipoproteins have been shown to play a direct role in virulence-associated functions, such as colonization, evasion of host defences and immunomodulation (Hutchings & Willett 2009; Khandavilli *et al.,* 2008). In Gram-negative bacteria, two of the three lipoproteins biosynthetic enzymes appear to be essential for viability (Gan *et al.,* 1993; Paitan *et al.,* 1999; Tjalsma *et al.,* 1999).

There are about 100 OM lipoproteins identified in *E. coli* (Miyadai *et al.,* 2004). Nearly all of the proteins in the OM assume a β-barrel conformation (Silhavy *et al.,* 2010). These proteins are β-sheets that are wrapped into cylinders (Silhavy *et al.,* 2010). Some of these OMPs such as the porins, OmpF, and OmpC function to allow passive diffusion of small molecules (mono- or disaccharides and amino acids) across the OM (Cowan *et al.*, 1992).

5.1.3 Proteomic approaches for analysis of virulence

In the pre-genomic era, systematic identification of virulence factors was routinely done employing biochemical approaches. The development of novel molecular approaches, such as genomics, transcriptomics and proteomics, has accelerated the speed with which we can identify virulence factors (Wu *et al.,* 2008). Among the various novel approaches, gel based techniques, such as proteomic techniques are of great use to investigate the protein-based virulence factors of pathogenic bacteria. In addition, it also aids in identification and characterization of proteins belonging to separate compartments of bacterial systems. The knowledge gained from the proteomic analysis helps us in deciphering the precise role and function each and every protein components (Wu *et al.,* 2008; Lee *et al.*, 2008).

5.1.3.1 Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS- PAGE)

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a type of electrophoretic technique used mainly to characterize and/or separate proteins and nuclide acids according to their molecular size (Laemmli, 1970; Sambrook & Russell, 2001). Electrophoresis, as the name implies, is the process of movement of charged molecules in a predefined medium under the influence of an electric field (Magdeldin, 2012). The mobility of particles in an electric field mainly depends upon the following factors; charge, shape and size of the molecules (Westermeier, 2005). SDS, as an anionic detergent, masks the charge of the proteins and gives a net negative charge per unit mass, such that the proteins move along the gel solely based on their molecular weight (Garfin 2009; Maizel 2000). **Figure 5.2** shows the basic methodology and principle of protein separation in SDS-PAGE.

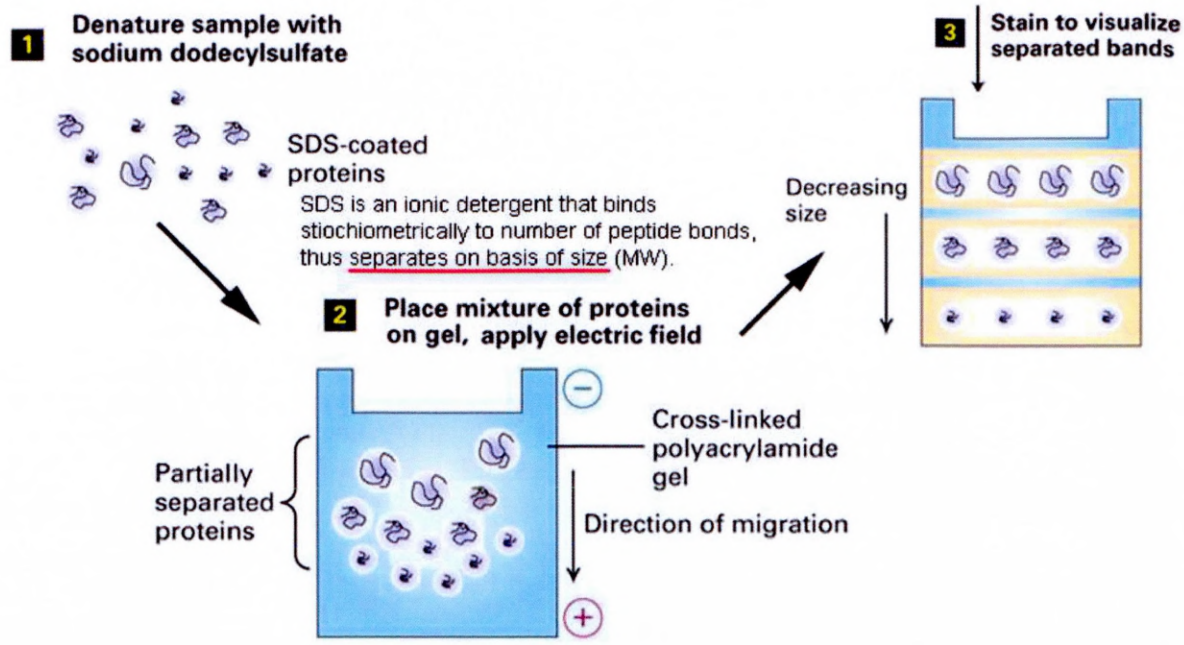

Figure 5.2: SDS-PAGE. Protein samples are loaded into the separate wells of the gel. Under electric charge the negatively charged SDS-protein complexes migrate in the direction of the anode, at the bottom of the gel. The sieving action of the porous polyacrylamide gel separates proteins according to their size.
(www.chem.fsu.edu/chemlab/bch4053l/character/mwdetermination/background.html) (Accessed on 20/11/2015) (Department of Chemistry and Biochemistry 2015).

5.1.4 SDS-PAGE for outer membrane protein analysis

Protein samples are loaded into the separate wells of the gel. Under the influence of an electric field the negatively charged SDS-protein complexes migrate in the direction of the anode, at the bottom of the gel. Subsequently, the sieving action of the porous polyacrylamide gel separates proteins according to their size.

OMPs play a key role in the pathogenicity, physiology and virulence of Gram-negative bacteria (Nikaido, 1994). The expression of these proteins change constantly in response to the variations in the environmental growth conditions they are exposed to (Puente *et al.,* 1991; Contreras *et al.,* 1995). Substantial changes in synthesis of their outer membrane proteins have been observed when bacteria are transferred to a new environment (Provenzano *et al.,* 2001;

Wibbenmeyer *et al.*, 2002). Effective changes in outer membrane protein thus equate to increased survival rate and pathogenicity. SDS-PAGE can thus be used to track and identify these changes.

5.1.5 Genomic characterization of virulence

During the last two decades the incidence of virulent multidrug-resistant (MDR) Gram-negative bacteria has increased considerably to become a significant health problem (Moxon & Tang 2000). Antibiotic resistance generally evolves through a series of complex interactions and resistance arises by mutation under clinical antibiotic selective pressure or via possession of mobile genetic elements from the environment over time (Landecker, 2015). The reservoir of such genes in the environment is a result of mixing of naturally occurring resistance in bacterial pathogens and selective effects of environmental pollutants, which can aid the bacteria in acquisition of multiple resistant genes (Wellington *et al.*, 2013). The identification of genes that provide certain traits to the bacterial pathogens has been facilitated by the development of molecular tools to search for genes that may be indispensable for virulence (Moxon & Tang, 2000). The search for the molecular genetic basis of virulence has led to the development of technology to look for multiple genes that may ultimately shed light on the regulation of virulence at molecular level (Salama & Falkow 1999; DiRita *et al.*, 2000; Fenwick 1990; Unsworth & Holden 2000). Reverse transcriptase PCR is one such methodology.

5.1.6 Reverse transcriptase PCR

Reverse transcriptase PCR (RT-PCR) has revolutionized the way clinical microbiologist detect the presence and level of expression of bacterial virulence genes (Coutard *et al.*, 2005). High sensitivity and high specificity are the advantage of RT-PCR technology over the conventional culture-based methods and PCR. PCR is amplification of a specific DNA sequence *in vitro* (Da

Costa, 1998). This process comprises of many cycles of template denaturation, primer annealing, and primer elongation to amplify DNA sequences (Santos & Ochman, 2004). Reverse transcriptase PCR has a similar principle as that of PCR. Reverse transcription is a process by which single stranded RNA (ssRNA) is transcribed into complimentary DNA (cDNA). The template used for this is total cellular RNA and the enzyme used for amplification is reverse transcriptase enzyme. This is the major difference between conventional PCR and reverse transcriptase PCR. In conventional PCR the template used is DNA and the polymerase enzyme used is DNA polymerase. Thus, RT-PCR combines cDNA synthesis from RNA templates with PCR to provide a rapid, sensitive method for analysing gene expression (Santos & Ochman, 2004). The ability to convert mRNA to DNA *in vitro* has made RT-PCR possible. The reverse transcriptase enzyme was discovered and isolated independently by Tenim and Baltimore in 1970 (Baltimore, 1970; Temin & Mizutani, 1970). **Figure 5.3** diagrammatically represents the synthesis of complementary DNA (cDNA) from mRNA by reverse transcriptase enzyme. Synthesis and quantification of cDNA from the mRNA of the bacterial cells can thus be used to identify and analyse the differences in level of expression of various genes in bacteria that are exposed to different conditions.

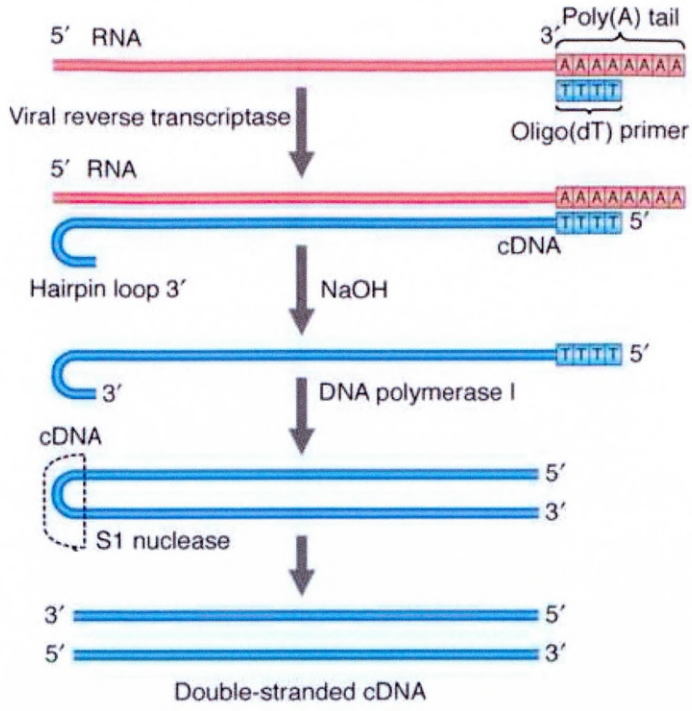

Figure 5.3: Schematic diagram showing the synthesis of double stranded cDNA from mRNA by reverse transcriptase enzyme (https://www.mun.ca/biology/scarr/MGA2-08-04.html , Accessed on 20/11/2015) (Steven, 2012).

5.2 Experimental approach

The main objective of this chapter is to characterize and compare various bacterial outer membrane proteins of test microorganisms. Standard laboratory strains where used as controls to see what difference the changes in environmental condition will bring upon on the outer membrane proteins of pathogenic bacteria belonging to the same species.

SDS-PAGE was employed to achieve this objective. The results from protein profiling will provide us this with information, such as total number of different proteins present and molecular weight of the proteins. The similarity/differences observed may help to identify whether there is variation among proteins with changes in thriving conditions. The next step was to characterize/compare and analyse the level of expression of specific virulence genes in selected Gram-negative pathogens isolated from the environment with the laboratory bacterial

strains that are pre characterized. RT-PCR as the main analytical tool used to achieve this objective.

5.2.1 Bacterial cultures

Five pairs of bacterial species consisting of previously described environmental isolates and their corresponding laboratory strain were selected for this study. *Burkholderia cepacia*, *Enterobacter agglomerans, Aeromonas hydrophila*, *Enterobacter* spp. and *Pseudomonas stutzeri* were the bacterial species included. The culture collection codes for laboratory strain of test organisms are as follows: *Burkholderia cepacia* (NCIMB13694), *Enterobacter agglomerans* (NCIMB 9680), *Aeromonas hydrophila* (NCIMB9239), *Enterobacter* spp. (NCIMB14479) and *Pseudomonas stutzeri* (NCIMB10783).

5.2.1 Outer membrane protein extraction

The outer membrane proteins of the test organisms were isolated using sarcosine as a solubilizing agent. The precise protocol followed for isolation of outer membrane protein is as described in **Chapter 2** section **2.12.**

5.2.2 Protein estimation

Protein concentration was calculated according to the method of Bradford (Bradford, 1976) using bovine serum albumin (BSA) as the standard and the concentration was expressed in mg/mL. Standard graph was plotted and the slope was calculated **Figure 5.4**. Refer **Chapter 2** section **2.12.1** for the procedure followed for estimation of protein concentration.

5.2.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) of purified phage proteins was performed for evaluating the nature of the isolated protein using vertical slab electrophoresis (BioRad, USA) by the method of Laemmli (1970).The methodology followed preparation of stacking gel/ resolving gel, sample loading and gel running parameters as described in detail in section **2.12.2** of **Chapter 2.**

5.2.4 Gel staining

Coomassie blue and silver staining of the gel after electrophoresis was performed by the methodology as described in sections **2.12.3** and **2.12.4** respectively of **Chapter 2.**

5.2.5 Molecular Weight Determination

The molecular weights of separated proteins are determined by measuring the relative migration distance (*Rf*) of the protein standards and the test protein. The details of calculation performed in order to determine the molecular weight is described in section **2.12.5** of **Chapter 2.**

5.2.6 Preparing bacterial cultures for RNA extractions

Tris-EDTA (TE) buffer (30mM Tris-HCl, 1mM EDTA, pH 8) containing 15mg/mL of lysozyme and 10µl of RNA-protect-reagent was pipetted into 15mL tubes. RNA protect reagent provides immediate stabilization of RNA from bacterial cells. Five mL of each bacterial culture were added to it and the mixture was vortexed (Fisons scientific equipment sgp-202-010J) for 5 seconds. Then it was incubated at room temperature for 5 minutes. After incubation the cultures were centrifuged at 8000rpm for 10 minutes (Eppendorf 5417 R). The supernatant was decanted completely and the pellet was kept for further processing.

5.2.7 RNA extraction and purification

The bacterial pellet obtained after the lysozyme and RNA-protect-reagent treatment was used directly for extraction of RNA. RNA was extracted using trizol reagent. Trizol reagent is a monophasic solution of phenol and guanidine isothiocyanate. During sample homogenization or lysis. TRIZOL reagent maintains the integrity of the RNA, while disrupting cells and solubilizing cell components. Isolation of RNA was followed by purification of the extracted RNA. Purification of total RNA from bacterial lysate was done using the RNeasy Mini Kit and DNase digestion was performed using QIAGEN DNase digestion by RNase-free DNA set following the manufacturer's instruction. The procedure followed for RNA extraction and purification is as given in **Chapter 2** section **2.13.**

5.2.8 cDNA synthesis

The cDNA was synthesized by reverse transcribing total RNA using RT2 First Strand Kit, Qiagen following the manufacturer's instructions. Refer to section **2.15** of **Chapter 2** for the protocol followed for cDNA synthesis.

5.2.9 Design of primers

Four pairs of primers were designed using the conserved stretches of the respective virulence genes using Primer3 software (http://simgene.com/Primer3. Accessed 11/04/14) (Primer3, n.d.). The methodology used design the primers are described in detail in section **2.15.2** of **Chapter 2.** The primer sequence and the expected amplicon sizes are as given in **Table 5.1.**

Table 5.1: List of primers designed for amplifying specific virulence genes from *Pseudomonas stutzeri*, *Enterobacter agglomerans*, *Burkholderia cepacia*, Enterobacter spp. and *Aeromonas hydrophila* and their estimated amplicon size.

Primer	Sequence of primers	Organism	Amplicon size	Ref.
OprC	CACCTTGCGATGTTCACTG TACGCCGATCACATCATGG	*P. stutzeri*	213bp	This work
TetC	GCGGTCAACTCATCCATG GCCACGTATGTCAAACGG	*E. agglomerans*	211bp	This work
Cation multidrug	AGGCTGACAACGAAAACGC GTTTCGCACAGCTGAAGAC	*B. cepacia*	265bp	This work
TetC	GCGGTCAACTCATCCATG GCCACGTATGTCAAACGG	*Enterobacter* spp.	211bp	This work
AheB	CTCTATGACAGGGGCATGG GAAATAGCGCATCACCAGC	*A. hydrophila*	233bp	This work
RecA	CACGATGTCATTTGGTCACG TGAGATGCGTTGACAAGTCC	Housekeeping genes	154bp	Landete *et al.*, 2010
RecG	GTCATGGCAATTTTTGATGC ATTCCTTGCTCATTTTTGGG	Housekeeping genes	128bp	Landete *et al.*, 2010

5.2.10 PCR

The primers as listed in **Table 5.1** were used to amplify the cDNA obtained from all the bacterial strains. The primers were diluted from the standard to required working concentration accordingly. Amplifications of cDNA were carried out in a thermal cycler (BioRad C1000). Five µL of the PCR product samples were analysed by electrophoresis on 1.5 % agarose. The PCR was performed in triplicate for each of the strains and each of the amplified genes used and presence of amplicons were visualized by agarose gel electrophoresis. The procedure followed for the preparation of the PCR master mix and the PCR program is as given in **Chapter 2** section **2.15.3.**

5.3 Results

5.3.1 Protein extraction and estimation

Proteins were extracted successfully following both the total protein method and outer membrane protein method. After protein extraction, the concentration of each sample was determined following Bradford method (Bradford, 1976). **Figure 5.4** shows the standard graph obtained using BSA as standard.

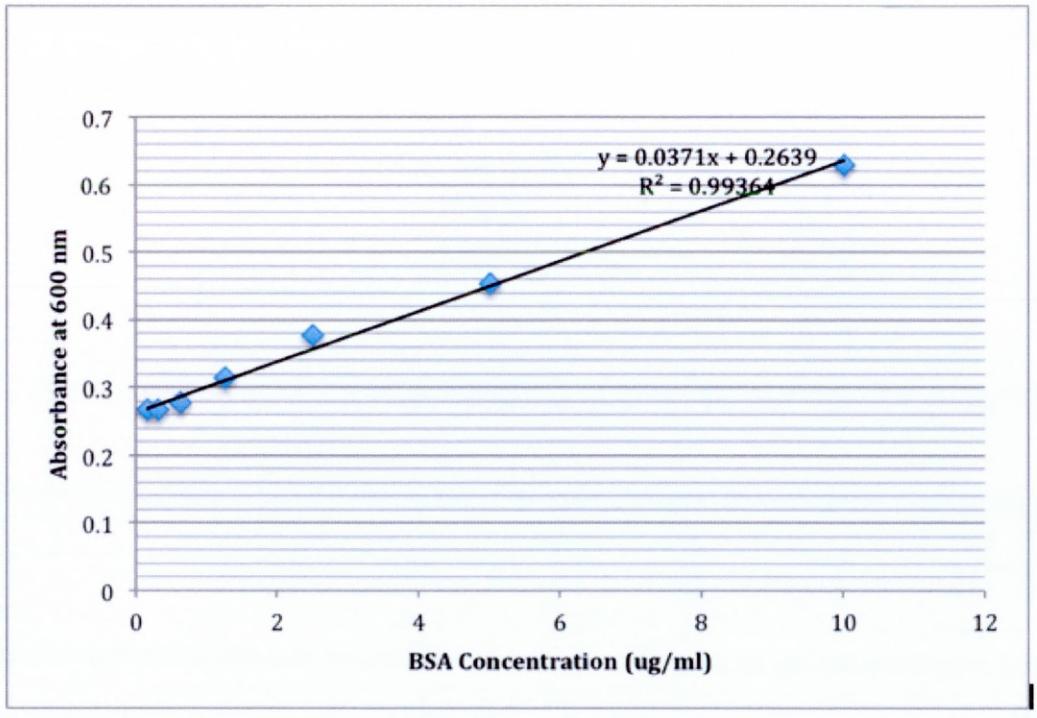

Figure 5.4: Standard graph for protein estimation, plotting BSA concentration (µg/mL) and its corresponding absorbance at 600nm. A marked scatter plot was drawn using Excel to obtain y and R2 values.

From the absorbance value of the test proteins, the concentration of proteins was calculated. The results of protein concentration calculation, estimation and volume required to equalize their concentrations for carrying out SDS-PAGE is calculated as given below in **Tables 5.2** and **5.3**.

Table 5.2: Calculation of protein concentration of the isolated outer membrane protein using the equation: y = 0.0371x + 0.2639, X = (y-0.2639) / 0.0371, y = Sub-mean of OD. Where; OD^1 = Optical Density replicate 1; OD^2 = Optical Density replicate 2, Mean = mean of OD 1 and OD2.

Sample	1	2	3	4	5	6	12	14
OD^1	0.40	0.25	0.28	0.29	0.33	0.26	0.34	0.301
OD^2	0.40	0.27	0.31	0.27	0.28	0.27	0.30	0.295
Mean	0.40	0.26	0.29	0.28	0.30	0.27	0.32	0.298
Sub-mean	0.39	0.25	0.28	0.27	0.2	0.25	0.3	0.28
Error	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Concentration (µg/µL)	7.17	4.645	5.24	4.96	5.39	4.73	5.76	5.21

Table 5.3: Results of protein concentration estimation and determination of required volume for preparing samples with uniform concentration for SDS-PAGE analysis. Where; Conc. = Concentration; LDS = *Lithium dodecylsulfate*; PBS = *Phosphate-buffered saline*; Prot. = protein.

Samples	Conc. (µg/µL)	St. Water (µL)	LDS (µL)	PBS top-up (µL)	LDS+Prot (µL)	FINAL total (µL)
Burkholderia cepacia (environmental isolate)	9.3	27.0	25	48.0	52.0	100.0
Burkholderia cepacia (laboratory strain)	7.9	31.5	25	43.5	56.5	100.0
Enterobacter agglomerans (environmental isolate)	6.6	37.8	25	37.2	62.8	100.0
Enterobacter agglomerans (laboratory strain)	5.8	43.1	25	31.9	68.1	100.0
Aeromonas hydrophila (environmental isolate)	5.8	43.3	25	31.7	68.3	100.0
Aeromonas hydrophila (laboratory strain)	5.2	48.4	25	26.6	73.4	100.0
Enterobacter spp. (environmental isolate)	4.8	51.9	25	23.1	76.9	100.0
Enterobacter spp. (laboratory strain)	4.0	62.5	25	12.5	87.5	100.0
Pseudomonas stutzeri (environmental isolate)	3.7	66.8	25	8.2	91.8	100.0
Pseudomonas stutzeri (laboratory strain)	3.4	72.9	25	2.1	97.9	100.0

5.3.2 SDS-PAGE

The result of SDS-PAGE of proteins extracted from environmental isolates as well as standard laboratory strains of *P. cepacia*, *E. agglomerans, A. hydrophila*, *Enterobacter* spp. and *P. stutzeri* is as shown in **Figure 5.5**.

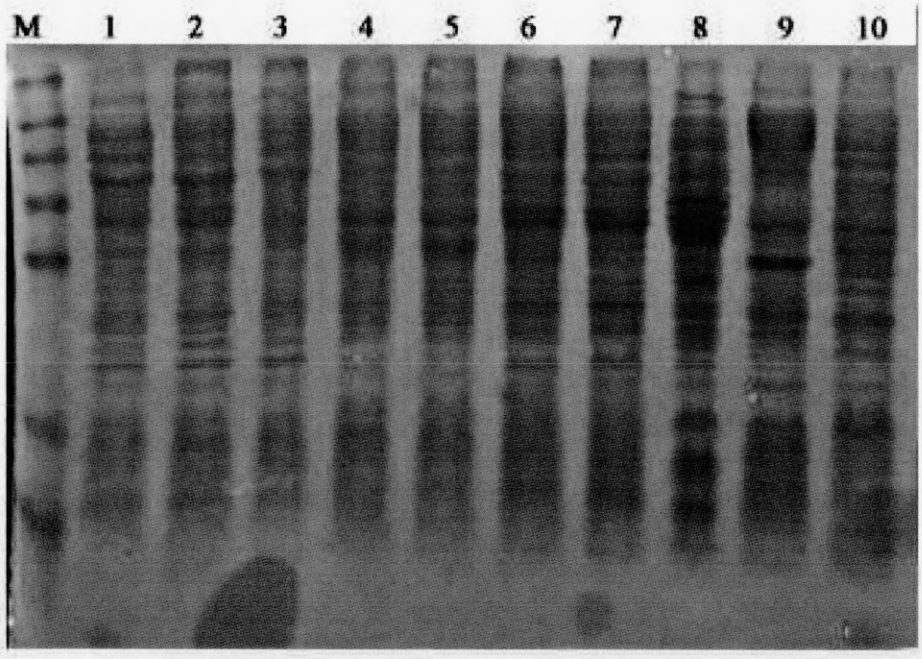

Figure 5.5: SDS-PAGE of outer membrane protein: Lane M = protein molecular weight marker; lane 1 = *Burkholderia cepacia* (environmental isolate); lane 2 = *Burkholderia cepacia* (laboratory strain); lane 3 = *Enterobacter agglomerans* (environmental isolate); lane 4 = *Enterobacter agglomerans* (laboratory strain); Lane 5 = *Aeromonas hydrophila* (environmental isolate); lane 6 = *Aeromonas hydrophila* (laboratory strain); lane 7 = Enterobacter spp. (environmental isolate); lane 8 = Enterobacter spp. (laboratory strain); lane 9 = *Pseudomonas stutzeri* (environmental isolate); lane 10 = *Pseudomonas stutzeri* (laboratory strain).

Lane 1 is protein molecular weight marker used to compare and estimate the molecular weight of the test proteins. Lanes 2 and 3 correspond to proteins extracted from environmental isolate and

standard strain of *P. cepacia*. Lane 4 and 5 correspond to proteins extracted from environmental isolate and standard strain of *E. agglomerans*. Lane 6 and 7 correspond to proteins extracted from environmental isolate and standard strain of *A. hydrophila*. Lane 8 and 9 correspond to proteins extracted from environmental isolate and standard strain of *Enterobacter* spp. and lane 10 and 11 correspond to proteins extracted from environmental isolate and standard strain of *P. stutzeri*.

5.3.3 Molecular weight estimation

The molecular weight of the separated proteins was calculated using *Rf* value measured using protein marker as standard. **Figure 5.6** shows the Rf values of markers plotted against Log10 molecular weight values. A linear trendline was applied and the graph equation was calculated as $y = -2.3855x + 2.2647$.

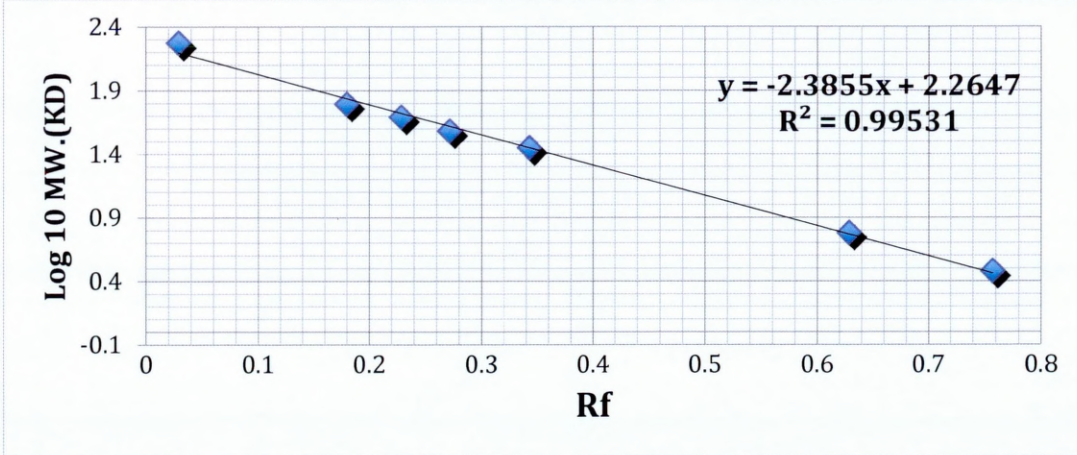

Figure 5.6: Standard curve. Rf values of markers were plotted against Log10 molecular weight values. A linear trend line was applied and the graph equation was calculated as y = –2.3855x + 2.2647.

The *Rf* is as given in **Table 5.4**. Molecular weight of outer membrane proteins were then calculated using equation $MW = 10^y$, where x = Rf of unknown protein. By substituting Rf value

(x) of each bands to the equation, $MW=10^{-2.3855x + 2.2647}$, the molecular weight can be calculated. The result of molecular estimation is as shown in **Table 5.5**.

In the case of the environmental isolate of *B. cepacia* 13 bands were present with molecular weights varying from 109.40 to 4.32 and for laboratory strain, 12 bands were present with molecular weight ranging from 118.11 to 3.71. In case of *E. agglomerans* environmental isolate 8 bands appeared with molecular weights ranging from 93.80 to 3.71, where as in case of its laboratory counterpart the total number of bands was found out to be 11 with molecular weights ranging from 101.30 to 4.67. The next group under study, *A. hydrophila* showed the presence of 13 and 14 bands for environmental and laboratory strain respectively. The outer membranes of *Enterobacter* spp. were found to comprise of 14 bands for both environmental isolates and laboratory strains. The protein profiling of the last group under study, *P. stutzeri* revealed presence of 13 bands in both environmental isolates and laboratory strains.

Table 5.4: Rf measurements of standard molecular weight marker and protein extracted from environmental isolates and standard strains for *Burkholderia cepacia*, *Enterobacter agglomerans*, *Aeromonas hydrophila*, Enterobacter spp. and *Pseudomonas stutzeri*. Where; EI = Environmental isolate; LS = Laboratory strain; - = no band.

Band No:	Marker	*B. cepacia* bands (*Rf*)		*E. agglomerans* bands (*Rf*)		*A. hydrophila* bands (*Rf*)		*Enterobacter* spp. bands (*Rf*)		*P. stutzeri* bands (*Rf*)	
		EI	LS	EI	LS	EI	LS	EI	LS	EI	LS
1	0.03	0.09	0.07	0.11	0.10	0.09	0.09	0.11	0.11	0.11	0.13
2	0.18	0.13	0.16	0.16	0.14	0.14	0.13	0.14	0.16	0.16	0.17
3	0.23	0.16	0.19	0.19	0.19	0.17	0.17	0.17	0.19	0.20	0.23
4	0.27	0.17	0.20	0.24	0.02	0.19	0.21	0.20	0.21	0.23	0.26
5	0.34	0.20	0.24	0.31	0.31	0.26	0.26	0.23	0.24	0.26	0.29
6	0.63	0.30	0.29	0.49	0.37	0.31	0.33	0.33	0.29	0.29	0.39
7	0.76	0.36	0.31	0.61	0.41	0.36	0.39	0.44	0.34	0.34	0.44
8	-	0.41	0.47	0.71	0.47	0.46	0.44	0.47	0.40	0.40	0.47
9	-	0.46	0.53	-	0.50	0.49	0.50	0.51	0.43	0.44	0.53
10	-	0.51	0.60	-	0.59	0.53	0.53	0.54	0.46	0.49	0.56
11	-	0.54	0.66	-	0.67	0.60	0.54	0.59	0.49	0.53	0.59
12	-	0.61	0.71	-	-	0.66	0.59	0.61	0.53	0.59	0.63
13	-	0.69	-	-	-	0.73	0.61	0.64	0.56	0.63	0.69
14	-	-	-	-	-	-	0.66	0.69	0.5	-	-

Table 5.5: Molecular weight determination of protein extracted from environmental and standard isolates for *Burkholderia cepacia*, *Enterobacter agglomerans*, *Aeromonas hydrophila*, Enterobacter spp. and *Pseudomonas stutzeri* using equation MW = 10^{y}, where x = Rf of unknown protein. By substituting Rf value (x) of each bands to the equation, $MW=10^{-2.3855x + 2.2647}$, the molecular weight was calculated. Where; EI = Environmental isolate; LS = Laboratory strain; MW = Molecular weight; kD = kilo Daltons; - = no band.

Band No:	Marker	MW *Burkholderia cepacia* bands (kD)		MW *Enterobacter agglomerans* bands (kD)		MW *Aeromonas hydrophila* bands (kD)		MW *Enterobacter* spp. bands (kD)		MW *Pseudomonas stutzeri* bands (kD)	
		EI	LS	EI	LS	EI	LS	EI	LS	EI	LS
1	188	109.40	118.11	93.80	101.30	109.40	109.40	93.80	93.80	93.80	86.86
2	62	86.86	74.47	74.47	80.43	80.43	86.86	80.43	74.47	74.47	68.96
3	49	74.47	63.86	63.86	63.86	68.96	68.96	68.96	63.86	59.13	50.70
4	38	68.96	59.13	46.95	155.48	63.86	54.75	59.13	54.75	50.70	43.47
5	28	59.13	46.95	31.96	31.96	43.47	43.47	50.70	46.95	43.47	37.27
6	6	34.51	37.27	12.70	23.50	31.96	29.59	29.59	37.27	37.27	21.76
7	3	25.38	31.96	6.36	18.66	25.38	21.76	16.00	27.40	27.40	16.00
8	-	18.66	13.72	3.71	13.72	14.81	16.00	13.72	20.15	20.15	13.72
9	-	14.81	10.08	-	11.76	12.70	11.76	10.89	17.27	16.00	10.08
10	-	10.89	6.86	-	7.41	10.08	10.08	9.34	14.81	12.70	8.65
11	-	9.33	5.05	-	4.67	6.86	9.34	7.41	12.70	10.08	7.41
12	-	6.35	3.71	-	-	5.05	7.41	6.36	10.08	7.41	5.89
13	-	4.32	-	-	-	3.44	6.36	5.45	8.65	5.89	4.33
14	-	-	-	-	-	-	5.05	4.33	7.41	-	-

5.3.4 RNA isolation

The result for agarose gel electrophoresis of the RNA isolated from environmental isolates and standard laboratory strains of *P. stutzeri*, *E. agglomerans, B. cepacia, Enterobacter* spp. and *A. hydrophila* is as shown in **Figure 5.7.**

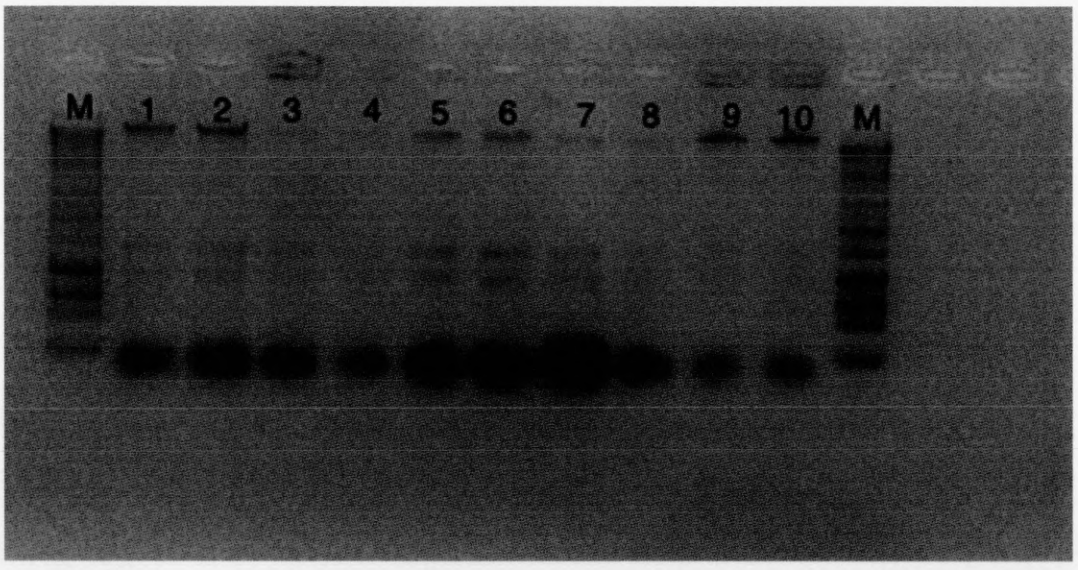

Figure 5.7: Agarose gel electrophoresis of the isolated RNA products. Lane M = 1 kb molecular weight marker ladder; lane 1 = *Pseudomonas stutzeri* (environmental isolate); lane 2 = *Pseudomonas stutzeri* (laboratory strain); lane 3 = *Enterobacter agglomerans* (environmental isolate); lane 4 = *Enterobacter agglomerans* (laboratory strain); lane 5 = *Burkholderia cepacia* (environmental isolate); lane 6 = *Burkholderia cepacia* (laboratory strain); lane 7 - Enterobacter spp. (environmental isolate); lane 8 = Enterobacter spp. (laboratory strain); lane 9 = *Aeromonas hydrophila* (environmental isolate); lane 10 = *Aeromonas hydrophila* (laboratory strain).

5.3.5 PCR Amplification of virulence genes

The primers designed from conserved stretches of virulence genes for the bacteria were successful in amplification of specific genes. The agarose gel electrophoresis of the PCR product of environmental isolates as well as standard laboratory strains of *B. cepacia*, *E. agglomerans, A. hydrophila*, *Enterobacter* spp. and *P. stutzeri* is as shown in **Figure 5.8**.

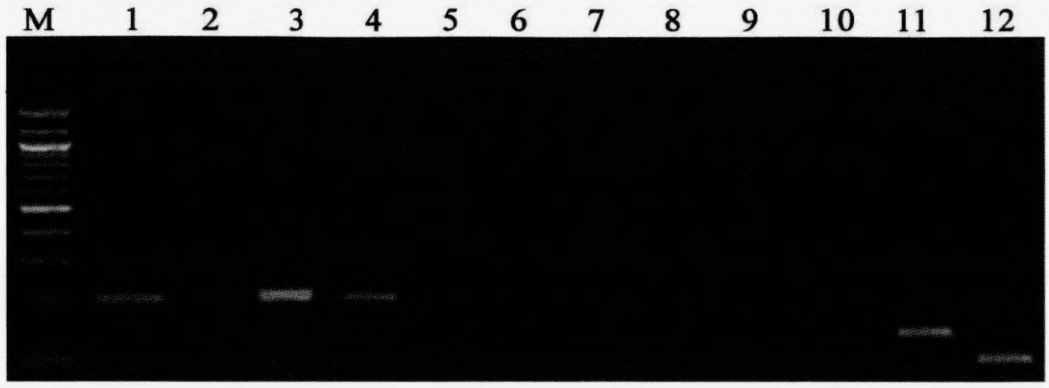

Figure 5.8: Agarose gel electrophoresis of the PCR product of virulence. Lane M = 1 kb molecular weight ladder; lane 1 = *Pseudomonas stutzeri* (environmental isolate); lane 2 = *Pseudomonas stutzeri* (laboratory strain); lane 3 = *Enterobacter agglomerans* (environmental isolate); lane 4 = *Enterobacter agglomerans* (laboratory strain); lane 5 = *Burkholderia cepacia* (environmental isolate); lane 6 = *Burkholderia cepacia* (laboratory strain); lane 7 = *Enterobacter* spp. (environmental isolate); lane 8 = *Enterobacter* spp. (laboratory strain); lane 9 = *Aeromonas hydrophila* (environmental isolate); lane 10 = *Aeromonas hydrophila* (laboratory strain); lane 11 = housekeeping gene (control); lane 12 = housekeeping gene (control).

Lane M is 1 kb molecular marker used to compare and estimate the molecular weight of the amplified PCR products. Lanes 1 and 2 correspond to *OprC* gene amplified from environmental isolate and laboratory strain of *P. cepacia.* An amplicon was observed at the expected range of 213 bp in lane 1, whereas in case of lane 2 no band was observed. The results indicate that the *OprC* gene is highly expressed in environmental isolates of *P. stutzeri,* whereas in the laboratory strain it is not expressed at substantial levels.

Lanes 3 and 4 correspond to the tetracycline resistance gene amplified from environmental isolate and laboratory strain of *E. agglomerans.* The expected amplicon size was 211 bp and bands of that size appeared in both lanes. Thus *E. agglomerans* isolate from

environment isolate and laboratory strain expressed the tetracycline resistance gene. However, a markedly higher rate of expression was observed in case of the environmental isolate.

Lanes 5 and 6 correspond to cation multidrug gene amplified from environmental isolate and laboratory strain of *B. cepacia.* A virulence gene of size 265 bp was expected, however no bands appeared in the gel indicating the absence of the cation multidrug in both environmental isolate and laboratory strain of *B. cepacia.*

Lanes 7 and 8 is that of tetracycline resistance gene amplified from environmental isolate and laboratory strain of *Enterobacter* spp. The PCR amplicon of size 211 bp was expected however the absence of bands in both the lanes indicates the absence of tetracycline resistance gene in both environmental and laboratory strain.

Lanes 9 and 10 correspond to *AheB* gene extracted from environmental isolate and laboratory strain of *P. stutzeri.* Absence of amplicon products at the expected range (233bp) indicates the absence of *AheB* gene in environmental and laboratory strain of *P. stutzeri.*

The last two lanes 11 and 12 correspond to the *RecA and RecG* gene amplification products. Housekeeping genes *RecA* and *RecG* was used as an internal positive control for the PCR analysis.

5.3.6 Gene expression and quantification by Gel densitometry

The gel picture obtained after agarose gel electrophoresis was then analysed through Gel densitometry analysis using gel Doc^{TM} imager (Life Technologies, Carlsbad, CA, USA). The results obtained are as given **Table 5.6. Figure 5.9** depicts the diagrammatic representation of the results.

Table 5.6: Densitometric analysis of PCR amplicons to analyze and quantify gene expression of virulence genes from environmental isolates and laboratory strains for *Pseudomonas stutzeri, Enterobacter agglomerans, Burkholderia cepacia, Enterobacter spp. and Aeromonas hydrophila.* The level of expression is given in percentages.

Bands obtained in agarose gel electrophoresis of the PCR product of virulence genes from environmental isolates and standard laboratory strains (Figure 5.8)	**Gene expression based on intensity values for each pixel in each row of the lane**	**Percentage of gene expression**
Lane 1 - *Pseudomonas stutzeri* (environmental isolate)	11596.45	13.7%
Lane 2 - *Pseudomonas stutzeri* (laboratory strain)	1767.21	2.1%
Lane 3 - *Enterobacter agglomerans* (environmental isolate)	23738.49	28.1%
Lane 4 - *Enterobacter agglomerans* (laboratory strain)	10259.62	12.1%
Lane 5 - *Burkholderia cepacia* (environmental isolate)	1767.21	2.1%
Lane 6 - *Burkholderia cepacia* (laboratory strain)	1767.21	2.1%
Lane 7 - *Enterobacter* spp. (environmental isolate)	1767.21	2.1%
Lane 8- *Enterobacter* spp. (laboratory strain)	1767.21	2.1%
Lane 9 - *Aeromonas hydrophila* (environmental isolate)	1767.21	2.1%
Lane 10 - *Aeromonas hydrophila* (laboratory strain)	1767.21	2.1%
Lane 11 - housekeeping gene (control)	14397.28	17.0%
Lane 12 - housekeeping gene (control)	12103.47	14.3%

The *OprC* gene was found to be highly expressed in environmental isolate of *P. stutzeri,* with an expression rate of 11596.45, whereas in the case of its corresponding laboratory strain it was substantially low (1767.21). Markedly higher rate of gene expression was seen in the case of *E. agglomerans* environmental isolate, 23738.49. The level of gene expression was comparatively

low in case *E. agglomerans* laboratory strain (10259.62). Expression of cation multi drug virulence gene in *B. cepacia* was not found to be significant in both environmental isolate and laboratory strain. A base line expression level of 1767.21 was recorded for both of them. The level of expression of *tetC* gene in environmental isolate and laboratory strain of *Enterobacter* spp. was also found to be not significant. A base line expression level of 1767.21 was recorded for both of them. Similar result was obtained for *A. hydrophila* (environmental isolate and laboratory strain) also. There was not significant expression and the expression level was 1767.21 for both of them.

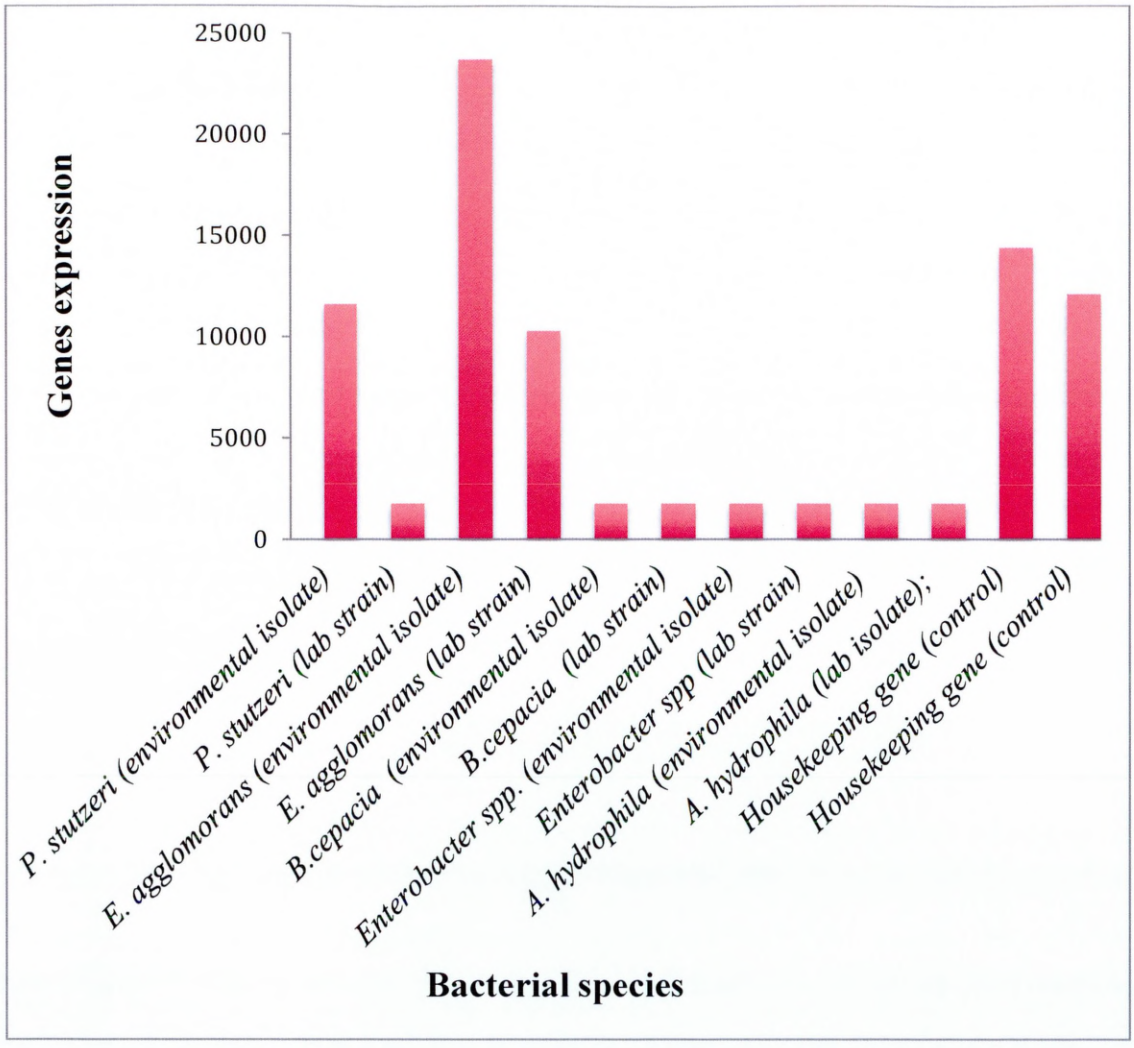

Figure 5.9: Graphic representation of gene expression of virulence genes from environmental isolates and laboratory strains for *Pseudomonas stutzeri*, *Enterobacter agglomerans, Burkholderia cepacia*, *Enterobacter* spp. and *Aeromonas hydrophila.*

The results of densitometric analysis of PCR amplicons to analyse and quantify gene expression of virulence genes from environmental isolates and laboratory strains for *P. stutzeri E. agglomerans, B. cepacia*, *Enterobacter* spp. and *A. hydrophila* was found to be in general agreement with the visual observation of agarose gel with PCR amplicons.

5.4 Discussion

The occurrence of Gram-negative bacterial mediated infections is on a rise and deserves special attention owing to multiple reasons. They have a very effective up-regulation or acquisition of genes that mediate antibiotic drug resistance, especially under antibiotic selective pressure (Donskey, 2006). Furthermore, bacteria have available to them a wide range of resistance mechanisms and often use multiple mechanisms to counter the same antibiotic and they can also use a single mechanism to affect multiple drugs (Peleg & Hooper, 2010b). According to the US National Healthcare Safety Network, Gram-negative bacteria account for about 30% of nosocomial (hospital-acquired) infections (Hidron *et al.,* 2008). The outer membrane of these organism have a major role in host pathogen interaction, e.g. during adherence, nutrient uptake from the host and overcoming defence mechanisms of the host during the establishment of an active infection (Seltmann & Holst, 2002). Therefore, the main objective of the present study was to compare and analyse the differences in outer membrane protein composition of various known Gram-negative pathogens of environmental origin and standard laboratory strains. The study microorganisms included in the work are *B. cepacia*, *E. agglomerans, A. hydrophila*, *Enterobacter* spp. and *P. stutzeri.*

SDS-PAGE is widely used for the analysis of bacterial outer membrane proteins (Dresler *et al.*, 2011). SDS-PAGE protein profiling aids in comparing closely related bacterial species and also help us to learn more about the protein biochemistry at the molecular level in an easy and rapid way. In addition to that, one-dimensional SDS-PAGE offers the combination of high-resolution and good reproducibility and therefore, is much better than lower resolving non-denaturing systems (Sambrook *et al.*, 1989).

Bacterial proteins are separated based on their molecular weight. Proteins with lower molecular weight will travel faster when compared to proteins of higher molecular weight. As a result, protein with small molecular weight will be at lowest position on the gel and larger molecular weight proteins will be at higher positions (Tuasikal & Wibawan, 2013).

In case of *B. cepacia* (isolate) a total of 13 types of proteins were isolated with molecular weight ranging from 109.396 kD to 4.32 kD. However, in case of the laboratory strain counterpart only 12 bands were present. A previous study by Zhou *et al.,* (2013) reported a similar finding in which *B. cepacia* exhibited altered protein profiling between wild-type and laboratory strains. The protein profiling of *E. agglomerans* also followed a similar trend with varying molecular weights. The total number of bands was also different with laboratory strain having more bands (11) as opposed to the environmental isolate with only 8 bands, ranging from molecular weight 93.799 kD to 3.71 kD. Similar results were obtained in a study conducted by Gayet *et al.,* (2003) on outer membrane protein of *Enterobacter aerogenes*. Where SDS-PAGE analysis of the outer membrane proteins showed a low level of expression of the major non-specific porin proteins in each of the clinical strains (strains 117 and 119) compared to that in reference strain ATCC 1304 of *Enterobacter aerogenes*. In the case of *Enterobacter* spp. protein profiling of both the environmental isolate and laboratory strain has same number of protein bands, but had a stark difference in the OMP pattern. Some of the proteins were found in higher concentrations, while others where present in lower concentrations, as evidenced by the thickness of the corresponding band. There were also subtle changes in the molecular weight of closely related proteins. The molecular weight of the environmental isolate of *Enterobacter* spp. varied from 93.799 kD to 4.327 kD, whereas in case of the laboratory strain the molecular weight varied from 93.799 kD to 7.413 kD. This finding is of special importance as the role of

Enterobacter's efflux pump mediated drug resistance has already been proved in this study (**Chapter 4**). It has been previously reported that in *E. aerogenes* drug resistance is acquired by the loss or alteration of a major porin protein or from altered porin expression in the constriction area, which decreases the diameter and modifies the electrostatic field or the expression of an efflux mechanism (Bornet *et al.,* 2000; Charrel *et al.,* 1996; Chevalier *et al.,* 1999; Dé *et al.,* 2001).

The protein profiling of *A. hydrophila* also showed a higher level of heterogeneity in banding patterns, with a total of 13 bands appearing in the environmental isolate (molecular weight ranging from 109.396 kD to 3.436 kD). 14 bands were observed in case of standard laboratory strain, with proteins having molecular weight range same as that of environmental isolate. The difference in banding pattern and absence of bands observed in case of *A. hydrophila* is in general agreement with the previous findings of Santos *et al.* (1996), Dooley & Trust (1988) and Kuijper *et al.* (1989). The variations in protein profiling of *A. hydrophila* can be attributed the fact that they are complex and diverse in their environmental condition of high selective pressure (Kuijper *et al.,* 1989).

The outer membrane protein analysis of *P. stutzeri* also revealed the variations in proteins between the environmental isolate and laboratory strain. A total of 13 prominent bands were observed in both the isolates, but prominent differences in concentration were noted for individual bands thicker bands, thinner bands and absence of bands. The results of comparison of these strains were similar to those reported in previous studies in *P. aeruginosa* (Goldberg & Ohman 1987; Goldberg *et al.,* 2000).

Thus, the proteomic analysis exhibits the marked variations in protein expression in bacterial strains of the same species under different environmental conditions. The next part of

this thesis was designed to confirm and quantify the differences in expression of various genes that are closely associated with bacterial virulence. RT-PCR was chosen as a methodology, which helps in accurately quantifying the mRNA representing each virulence related protein. In this study, real time PCR was used as a molecular tool to achieve to characterize/compare and analyse the level of expression of specific virulence genes in selected Gram-negative pathogens isolated from the environment with the laboratory bacterial strains. The major advantages of RT-PCR include high sensitivity, specificity and quantification of genes amplified (Bustin 2002; Gachon *et al.,* 2004). Gram-negative bacterial pathogens, which exhibited various drug resistances, were selected for the study. *E. agglomerans, A. hydrophila*, *Enterobacter* spp., *B. cepacia,* and *P. stutzeri* isolated from the river were included. Standard laboratory strains of *B. cepacia*, *E. agglomerans, A. hydrophila*, *Enterobacter* spp. and *P. stutzeri* were also included for comparative purposes. The virulence genes selected for each bacterial species are as follows:

OprC gene coding for the outer membrane protein was selected for screening and comparing its level of expression in *Pseudomonas* species. The result showed an increased level of expression of *OprC* in environmental strain of *P. stutzeri* when compared to that of the laboratory strain, thereby confirming its increased virulence. The marked increase in level of expression in environmental *P. stutzeri* might be due to the higher level of environmental stress faced by this organism. High levels of outer membrane protein expression equate to a better chance of survival and virulence.

TetC is the gene selected for screening virulence in *E. agglomerans* and *Enterobacter* spp. Resistance mechanisms encoded by tetracycline-resistant genes include energy dependent efflux, ribosomal protection and enzymatic inactivation (Roberts, 2005). Presently about 35 classes of tetracycline-resistant genes have been identified (Roberts, 2005). Major facilitator

superfamily (MFS) of efflux pumps for tetracycline operate by removing tetracycline in an energy-dependent fashion from bacterial cell (Chopra & Roberts, 2001). Tet pumps are classified into six groups based on amino acid sequence, with *Tet (A), Tet (B), Tet(C), Tet (D)* and *Tet(E)* placed in group 1 due to amino acid sequence similarity. *TetB* encodes a pump that is able to extrude both tetracycline and minocycline (Guay & Rothstein 1993; Petersen *et al.,* 1999). *TetC* was expressed substantially in both the environmental isolate and the laboratory strain of *Enterobacter agglomerans*. This observation confirms the importance of *TetC* in conferring resistance in *Enterobacter agglomerans*. The level of expression in environmental isolate was 23738.489, whereas in the case of laboratory strain it was comparatively low at 10259.619. This result also indicates the role of external physico-chemical stress faced by *Enterobacter agglomerans* in harsh environmental conditions forcing it to undergo effective mutations to survive and increase its virulence. These results are in agreement with the result obtained in the previous chapters of this thesis confirming the presence of various drug resistances in the isolated bacterial species. Cation multi-drug genes have been used to screen the virulence of *B. cepacia.* It is known to code for the efflux pumps capable for effectively eliminating various classes of antimicrobial compounds from the bacterial cell, promoting multidrug resistance phenotype (Magnet *et al.,* 2001; Piddock 2006).

The *aheB* gene was used to screen the level of virulence gene expression in *A. hydrophila*. *AheB* is known to encode a resistance-nodulation-cell division (RND) protein. It is responsible for aminoglycoside, fluoroquinolones, tetracyclines, chloramphenicol, erythromycin, trimethoprim and ethidium bromide resistance (Alekshun & Levy, 2007). The qPCR with cation multi-drug, *TetC* and *AheB* virulence genes for screening the virulence in *Enterobacter* spp., *A.*

hydrophila and *B. cepacia* only give base line level of expression suggesting the lack of significant expression of the genes in the respective bacterial groups.

5.5 Conclusions

In this study marked variation in expression of outer membrane proteins were observed in all of the isolates studied. The outer membrane plays an important role in making Gram-negative bacteria competitive when exposed to various harsh environmental conditions. It is the site where constant changes take place to make bacteria adapt to the ever changing novel environmental conditions. This notion has been proven in the present study where significant variation were seen when closely related bacterial group belonging to the same species exhibited different protein banding pattern. The studies employing RT-PCR also confirmed the findings of proteomic analysis. The level of gene expression can be analysed via quantifying the mRNA via RT-PCR. The concentration of mRNA is directly proportional to the rate of gene expression. Recent advances in molecular approaches have given environmental microbiologists an upper hand in analysing the activities of bacterial pathogens at a much appreciated finer level of gene expression (CEFIC, 2004). Real time PCR is such a tool. In the present study the enhancement and over expression of virulence genes in environmental isolates of medical importance, *P. stutzeri, E. agglomerans* was proven employing the RT-PCR method. This confirms the idea of enhanced drug resistance and survival of bacterial pathogens in natural environments.

Chapter 6. General Discussion

"It is not the strongest of the species that survives, nor the most intelligent, but rather the one most adaptable to change." (Megginson, 1963).

Present day bacteria fit directly into this famous description in gaining antibiotic resistance and surviving well enough in the antibiotic era. According to WHO "Anti-Microbial Resistance is a natural phenomenon accelerated by use of antimicrobial medicines" (WHO, 2014). The incidence of antibiotic resistance is ever increasing and does not show any signs of slowing down (Tang *et al.*, 2014). The achievements of modern medicine are put at risk by antimicrobial resistance. Without effective antimicrobials for prevention and treatment of infections, the success of organ transplantation, cancer chemotherapy and major surgery would be compromised (WHO, 2015).

It is estimated that approximately 5 to 12% of patients acquire an infection during their hospital stay in the European Union (EU), Norway and Iceland. Approximately 400,000 hospital patients in the EU contract a resistant strain and 25,000 die of the complication arising from these infections annually (World Health Organization 2015). The economic burden associated with management of antimicrobial resistant strain infections is tremendous. Multidrug-resistant (MDR) bacteria in the EU are estimated to cause an economic loss of more than €1.5 billion each year (World Health Organization 2015).

Antibiotic-resistance mechanisms in bacteria have evolved by two major pathways, (1) mutation (Martinez & Baquero, 2000) and (2) attainment of resistance genes from the natural environment by horizontal gene transfer (Davies, 1994). Among these two, horizontal gene transfer plays a major role in the rapid spread of resistance among microbial species (Martinez,

2009) and in most cases the antibiotic resistance genes originate in the environmental microbiota (Davies, 1997).

Thus, this study was designed to focus upon assessing the role of the natural environment in acting as a stage for the development of and a reservoir for antibiotic resistance genes and antibiotic-resistant pathogens. The thesis hypothesis was: The environment serves as a reservoir of antibiotic resistant bacteria and the Null hypothesis set was: The environment does not serve as a reservoir of the antibiotic resistant bacteria.

A river with an associated high anthropogenic and agricultural activity was chosen for the study to test the hypothesis. The presence of antibiotic resistance genes and pathogens in aquatic ecosystems has been proven by many scientists previously (Hamelin *et al.,* 2007; Servais & Passerat 2009; Stoppe *et al.,* 2014). The occurrence of antibiotic resistant bacteria in the natural environment can be due to a variety of reasons, including natural production of antibiotics by soil microbes, run-off from animal feed or crops contaminated with growth promoting antibiotics and from waste products originating from farm bred animals or humans (Boon & Cattanach, 1999; Davies, 1994; Levy, 1997; Tenover & McGowan, 1996; Witte, 1997, 1998). Recently a group of researchers have isolated fluoroquinolone and macrolide resistant *Campylobacter* spp. from the intestinal content and excreta of broiler chickens and swine from slaughter houses and conventional farms in China (Wang *et al.,* 2015). The situation is important and indicative of the bacterial trend towards widespread resistance, that researchers have concluded that these antibiotics are no longer suitable for the treatment of human campylobacteriosis in China (Wang *et al.,* 2015).

In our study membrane filtration techniques were used to isolate and analyze the microbiota of the water samples. The membrane bound microbes were then grown on a variety of nutrient agar media, under both aerobic and anaerobic conditions in order to isolate the maximum

number of bacteria belonging to both categories. A total of 146 bacterial isolates were isolated to pure culture and putatively identified using API strips. These isolates were subsequently tested for their antibiotic sensitivity (**Table 3.6**). The isolated strains were tested against a total of 16 antibiotics; chloramphenicol, erythromycin, fusidic acid, oxacillin G, novobiocin, penicillin G, streptomycin, tetracycline, ampicillin, cephalothin, colistin sulphate, gentamicin, streptomycin, sulphatriad, tetracycline and co-trimoxazole. A significant number of bacterial isolates exhibited antibiotic resistance against one or more of the selected antibiotics. Therefore, the hypothesis that the environment serves as a reservoir of antibiotic resistant bacteria was proven in this preliminary study.

Similar results were obtained in a study conducted in Victoria, South-Eastern Australia in *Escherichia coli* isolated from the Yarra River (Boon & Cattanach, 1999). The incidence of resistance towards ampicillin, chloramphenicol, kanamycin, nalidixic acid, neomycin and streptomycin and penicillin was found to be significant (Boon & Cattanach, 1999). In a separate study conducted by a group of researchers from the US, they reported similar observations (Ash *et al.,* 2002). They isolated antibiotic-resistant bacteria from 16 different rivers that were resistant to β-lactam and non-β-lactam antibiotics. The antibiotics tested in their study include cefotaxime, ceftazidime, amoxicillin (plus clavulanic acid), cephalothin, imipenem, kanamycin, streptomycin, chloramphenicol, tetracycline and ciprofloxacin. An estimate of 40% of the total bacteria isolated were found to be resistant to more than one antibiotic and had at least one plasmid (Ash *et al.,* 2002).

In this study, a total of 20 bacterial strains, which showed significant multidrug resistance were selected for further study. In addition to that, 13 isolates which possessed resistance towards penicillin G and tetracycline were also chosen for a drug inhibition assay in the 96-well plate system (**Table 3.6**). Four antibiotics chosen for the study included ampicillin, co-trimoxazole,

novobiocin and tetracycline. Ampicillin is a broad spectrum semi-synthetic derivative of penicillin that can be taken orally to combat Gram-positive and Gram-negative bacteria (Rafailidis *et al.*, 2007). Co-trimoxazole is a combination of drug used against both Gram-positive and Gram-negative organisms. It is found to be effective in the treatment of many infections, especially *Pneumocystis* pneumonia in AIDS patients (Suthar *et al.*, 2012). Novobiocin is a drug of choice for treating Gram-positive bacterial infections (Robson & Baddiley, 1977). Tetracycline is an aphthacene antibiotic that inhibits amino-acyl-tRNA binding during protein synthesis in bacteria. It is used against bacterial infections, especially pneumonia and other respiratory tract infections (PUB Chem, n.d.) Of the 33 bacterial isolates studied, five bacterial isolates exhibited resistance to all four antibiotics as evidenced by positive absorbance readings (450nm) in microtitre wells containing antibiotics at a concentration greater than 128μg/mL in the drug inhibition assay (**Table 3.7**). These five strains were then identified using 16S rRNA gene sequencing as *Enterobacter* spp., *Burkholderia cepacia, Aeromonas hydrophila*, *Enterobacter agglomerans* and *Pseudomonas stutzeri* (**Table 3.8**). These bacterial isolates were then subjected to further studies, which included screening for the presence of (i) plasmids, (ii) efflux pump, (iii) β-lactamase-mediated drug resistance, (iv) proteomic characterization, (v) comparison of outer membrane proteins of environmental isolates with laboratory strains and (vi) genomic characterization and comparison of virulence genes of environmental isolates with laboratory strains.

Enterobacter spp. are responsible for a substantial number of hospital infections attacking the lungs, urinary tract, intra-abdominal cavity and intravascular devices (Nazarowec-White & Farber, 1997). In one study, over 33,869 Gram-negative bacteria were isolated from intensive care units in the United States of which 16.1% comprised of *Enterobacter* spp. (Itokazu *et*

al.,1996). The isolated *Enterobacter* spp. exhibited marked resistance towards extended-spectrum cephalosporins (Itokazu *et al.*,1996).

Burkholderia cepacia is a Gram-negative bacterium, which is responsible for opportunistic infections in immuno-compromised patients or those who are hospitalized (Baylan, 2012). Patients with underlying respiratory diseases, like cystic fibrosis and chronic granulomatous disease are more prone to infection by *Burkholderia cepacia* (Matthaiou *et al.*, 2011). *B. cepacia* complex (Bcc) bacteria are known to exhibit multidrug resistance via innate and acquired mechanisms of resistance (Aaron *et al.,* 2000; Burns & Saiman 1999; Burns *et al.,* 1996; Nair *et al.,* 2004). In a study among cystic fibrosis patients with *Burkholderia cepacia* infection, 62 to 97% were found to harbour bacteria that were resistant towards antibiotics meropenem, ciprofloxacin, minocycline, trimethoprim-sulfamethoxazole and chloramphenicol (Zhou *et al.,* 2007).

The genus *Aeromonas* includes Gram-negative, rod shaped bacteria comprising several species, and are associated with the aquatic environment. *Aeromonas* spp. have been reported in cases of septicaemia, gastroenteritis and skin diseases among human (Joseph *et al.,* 2013). In a study conducted on *Aeromonas* species isolated from aquatic environments a significant number of them were found out to be resistant towards antibiotics vancomycin, ampicillin, co-trimoxazole, nalidixic acid, tetracycline and rifampicin and 20% of them scored a MAR index that was higher than 0.2, which is indicative of the high risk environment (Joseph *et al.,* 2013).

Pseudomonas stutzeri is an aerobic, Gram-negative bacterium. It is generally found as a saprophyte in soil and water. However, it occasionally results in serious community or hospital acquired infections (Lalucat *et al.,* 2006), mainly in immuno-compromised patients with underlying conditions or in those who have undergone surgery (Noble & Overman, 1994). *Pseudomonas stutzeri* are mainly isolated from such patients' surgical wounds, blood,

respiratory tract and urine samples (Holmes 1986; Taneja *et al.,* 2004). *Pseudomonas stutzeri* are generally susceptible to most of the antibiotics (Noble & Overman 1994; Tattawasart *et al.,* 1999) owing to their lower incidence of infection in humans and hence, their lower exposure to antibiotics (Lalucat *et al.,* 2006). However, it should be noted that drug-resistant forms may develop amongst clinical isolates and have been recovered for almost all antibiotic families (Lalucat *et al.,* 2006).

Therefore, the first objective of the study *"...to isolate and identify Gram-negative bacteria from a natural water environment that display multi-drug resistance"* has been successfully achieved. A total of 146 bacterial organisms were initially isolated from the river water sample and from them 33 of them were selected based on the antibiotic sensitivity assay. Among the 33 isolates, five bacterial isolates which exhibited multidrug resistance (MDR) in drug inhibition assay were identified and used for further study. Thus, it can be concluded from the first part of experimental proves the hypothesis of the thesis that environment as a reservoir of antibiotic resistant bacteria.

The presence of bacterial isolates exhibiting high levels of antibacterial drug resistance can be directly related to high anthropogenic activity (Ayandiran *et al.,* 2014). The incidence of bacterial resistance in river ecosystems is a highly dangerous situation, as the probability of people becoming infected by these organisms directly or indirectly through consumption of river fauna is significant (Schmidt *et al.,* 2001). The prevalence of intrinsic multi-resistance to common antibiotics has been previously reported (Wright 2007; Baltz 2008; Brown & Balkwill 2009; Thaller *et al.,* 2010).

The mechanism by which different bacterial species attain antibiotic resistance varies widely depending on the bacteria and the environment in which they are present. The three fundamental mechanisms by which bacteria achieve this are; (i) enzymatic degradation of

antibacterial drugs (e.g. the most prominent example being β-lactamase), (ii) alteration of bacterial proteins that are antimicrobial targets and (iii) through changes in membrane permeability to antibiotics (e.g. efflux pumps). Antibiotic resistance can be either plasmid-mediated or maintained on the bacterial chromosome (Dever, 1991). Therefore, in this study, the five selected bacterial species exhibiting multiple antibiotic resistance (MAR) were subjected to a series of experiments designed to exhibit whether the resistance they have acquired is plasmid-mediated, efflux pump mediated drug resistance or via the production of β-lactamase enzyme production.

Among various mechanisms, plasmid mediated drug resistance is of paramount importance as they aid in transference of non-conjugative and conjugative transposons housing resistance genes between different species and genera. Plasmids also play an important role in the dissemination of antimicrobial resistance genes within the gene pool in an environment (Wu *et al.*, 2014). The presence of plasmid mediated drug resistance was studied employing transformation experiments using DH5α *Escherichia coli.* Plasmids isolated from all the bacterial strains under study were transformed into the DH5α *E. coli* cells. The transformants were subsequently grown on ampicillin-containing LB agar plates. DH5α *E. coli* cells containing plasmids from *Burkholderia cepacia* and *Aeromonas hydrophila* grew in LB agar plates containing ampicillin at a concentration of 128μg/mL and 64μg/mL respectively indicating the role of plasmids in transfer of drug resistance (**Table 4.1**). The presence of plasmid-mediated drug resistance has been previously reported in *Burkholderia cepacia* isolated from brackish water aquaculture farms in India (Kumar & Surendran, 2006). Six strains exhibiting multidrug resistance when tested for the presence of plasmid showed that all the strains harboured three, high copy number small plasmids of size 4kb, 2kb and <2.0 kb. Plasmid based antibiotic resistance was proven via curing and transformation experiments (Kumar & Surendran, 2006).

Similarly the presence of plasmid mediated drug resistance in *Aeromonas hydrophila* was proven in a study on *Aeromonas hydrophila* isolates from skin lesions of the common freshwater fish, *Telapia mossambica* (Son *et al.,* 1997). Of the 21 fish isolates studied, seven of them harboured plasmids, with sizes ranging from 3 to 63.4 kb. Plasmid based antibiotic resistance was proven using an *Escherichia coli* recipient by single-step conjugation (Son *et al.,* 1997).

The presence of efflux pump mediated drug resistance was studied using the efflux inhibitor carbonyl cyanide m-chlorophenyl-hydrazone (CCCP). The results obtained suggest the presence of efflux pump mediated drug resistance in *Enterobacter agglomerans* and *Enterobacter* spp. There was a significant reduction in MIC for *Enterobacter agglomerans* and *Enterobacter* spp. where exposed to antibiotics in the presence of the efflux inhibitor. The presence of CCCP in the medium resulted in a reduction of minimum inhibitory concentration from 64 and 128μg/mL to 4μg/mL and 1μg/mL in case of *E. agglomerans* and *Enterobacter* spp. respectively (**Table 4.2**). Efflux via a membrane pump is a common antibiotic resistance (or tolerance) mechanism observed in Gram-negative and Gram-positive bacteria (Mahamoud *et al.,* 2007). It has been previously reported that multidrug resistant forms of *Enterobacter aerogenes*, possess altered O-polysaccharide and active efflux of chloramphenicol (Gayet *et al.,* 2003).

The presences of β-lactamase activity in the test organisms were determined using a β-lactamase inhibitor, clavulanic acid. No positive correlation was observed between the bacterial strains and their ability to survive in presence of β-lactamase inhibitor (**Table 4.3**). The result is suggestive of absence of β-lactamase production in all the bacteria under study.

Through these experiments the role of plasmid-mediated and efflux pump-mediated drug resistance has been proven in *Burkholderia cepacia*, *Aeromonas hydrophila* (plasmid-mediated) and *Enterobacter agglomerans* and *Enterobacter* spp. (efflux pump-mediated) (**Table 6.1**). The mechanism by which *Pseudomonas stutzeri* achieves drug resistance remains unclear at this stage

and further proteomic and genomic characterization experiments were conducted in these test organisms to learn more about the drug resistance at a much deeper level.

Table 6.1: The mechanisms of drug resistance in *Burkholderia cepacia*, *Enterobacter agglomerans*, *Aeromonas hydrophila*, *Enterobacter* spp. and *Pseudomonas stutzeri*.

Species	Plasmid-mediated antibiotic resistance	Efflux pump-mediated antibiotic resistance	β-lactamase production-mediated antibiotic resistance
Burkholderia cepacia	✓	X	X
Enterobacter agglomerans	X	✓	X
Aeromonas hydrophila	✓	X	X
Enterobacter spp.	X	✓	X
Pseudomonas stutzeri	X	X	X

The results of this table (6.1) are also in general agreement with the hypothesis of the thesis. The presence of bacteria harbouring plasmid-mediated and efflux pump-mediated resistance from the environmental isolates proves the hypothesis that the environment serves as a reservoir of antibiotic resistant bacteria, which have the ability to transfer their resistance mechanisms via horizontal and vertical transmission.

The proteomic characterization of the test strains comprised of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the outer membrane proteins of the test bacterial strains. The role of outer membrane proteins in multiple drug resistance has been previously described in *Pseudomonas aeruginosa*, where three different outer membrane proteins, OprM, OprJ and OprN are associated with multiple drug resistance (Masuda *et al.*,

1995). Similarly in the pathogenic bacteria *Klebsiella pneumonia*, imipenem resistance was found to be linked to the loss of an outer membrane protein (Bradford *et al.*, 1997). In the present study the outer membrane proteins of test strains were compared with those of their standard laboratory strains in order to determine the influence of the environment in moulding the closely related species for enhancing the drug resistance. Marked variations were observed in the case of the organisms tested, strongly suggesting the role of outer membrane proteins in conferring resistance in environmental isolates (**Figure 5.5**; **Table 5.5**). These results are also in favour of the tested hypothesis. It has been proven that the environment harbours antibiotic resistant bacteria exhibiting marked variations in membrane proteins as part of their attainment of antibiotic resistance.

Apart from the marked heterogeneity in the total number of bands, another major observation from the SDS-PAGE analysis was the marked variation in expression of outer membrane proteins isolated. Some of the proteins were found to be present in higher concentrations or lower concentrations when compared to their laboratory counterparts, as evidenced by variation in band density in SDS-PAGE gels. This was especially true in the case of *Enterobacter* spp. and *P. stutzeri.* Therefore, the next sets of experiments were designed to analyze the differences in expression of various genes that are closely associated with bacterial virulence. Reverse transcriptase PCR (RT-PCR) was used to study the differences in concentration of mRNA, which in turn helps to characterize and correlate differences in virulence gene expression. The total mRNA was isolated from all the environmental isolates and their corresponding laboratory strains and was then subjected to cDNA synthesis. Sets of five different primers corresponding to different virulence genes were designed to screen the test organisms. The virulence genes selected for the study included *OprC* (*P. stutzeri*), *Tetc* (*E. agglomerans* and *Enterobacter* spp.) *cation multidrug gene* (*B. cepacia*) and *AheB* (*A. hydrophila*). The results of

this experiment are also in agreement with the test hypothesis. Expression of virulence genes in the environmental isolates clearly indicates the role of the environment acting as a reservoir for multidrug resistant bacterial species (**Figure 5.8**, **Figure 5.9** and **Table 5.6**). The expression rate of environmental strain of *P. stutzeri* was 11596.45 and in the case of its laboratory counterpart it was only 1767.21. An increased level of expression of *OprC* gene, which codes for the outer membrane protein in environmental strain of *P. stutzeri*, is indicative of increased virulence. Variations in expression of outer membrane protein enable pathogens to enhance their virulence in stressful environmental conditions. The role of outer membrane proteins in providing multidrug resistance has been shown previously (Masuda *et al.,* 1995). Similarly, the presence and increased expression of *TetC* gene in the *E. agglomerans* environmental strain indicates the role of antibiotic polluted environment in increasing the expression of the tetracycline resistance gene. The environmental isolate recorded an expression rate of 23738.48, where as in case of its laboratory counterpart it was substantially lower (10259.62). The presence of the tetracycline resistance genes in *Enterobacter* spp. has been previously proved in bacterial populations isolated two apple orchards (Schnabel & Jones, 1999).

The findings of the present study are of great importance, especially in the wake of latest reports from China, where a group of scientists recently reported the emergence of the plasmid-mediated polymyxin resistance mechanism, MCR-1, in *Enterobacteriaceae* isolated from pigs. Polymixins are the last group of effective antibiotics against which there were no previous reports of plasmid-mediated drug resistance (Liu *et al.,* 2015). Scientists warn the possibility of spread of this antibiotic resistant gene and the present study further confirms this threat. The environment can act as breeding and fostering ground for this novel drug resistant genes eventually leading to their pan continental spread. Modern medicine will be forced to face and fight the advent of post antibiotic era if no coordinated steps are taken at a global level.

6.1 Conclusions

In the present study, the incidence of plasmid-mediated drug resistance and efflux pump-mediated drug resistance has been proven. In addition to that, role of various membrane proteins in drug resistance and the environment mediated modulation of protein expression to enhance the virulence and overall survivability has been indicated. The hypothesis tested in this study was whether the environment serves as a reservoir for antibiotic resistant bacteria or not. Interpretation of the results strongly supports the role of the environment, which in this case is an anthropogenically polluted river, in harbouring, nurturing and acting as a reservoir for various bacterial pathogens of medical importance.

Similar results were reported by group of researchers from Nigeria, where they have isolated multi drug resistant *Micrococcus* spp., *Streptococcus* spp., *Staphylococcus* spp., *Bacillus* spp. and *Pseudomonas* spp. from the anthropogenically polluted Oluwa River (Ayandiran *et al.*, 2014). Similarly, sediment and water samples from Liuxi River, China were found out to harbour antibiotic resistance genes (ARGs).The study concluded that the river environment was contaminated by antibiotics and proposed the role of river as a reservoir of ARGs (Xiong *et al.,* 2014). The Dighty Burn is a lotic ecosystem with a high degree of spatial and temporal heterogeneity. Thus, it can be further hypothesized that within the river ecosystem, the benthic region that is comparatively stable serves as the reservoir for antibiotic resistant bacteria. Non-pathogenic indigenous bacteria acquire antibiotic resistance genes through their encounter with pathogenic bacteria that are anthropogenically introduced. A good proportion of the pathogenic bacteria are attached to the particulate matter reach the benthic region of river ecosystem via sedimentation. The water column above the benthic region is periodically inoculated with antibiotic harbouring bacteria via resuspension of water column. The following figure (6.1) is the schematic representation of this hypothesis.

The environment is considered to be a *"melting pot of antimicrobial resistance"* (Da Costa *et al.*, 2013). This is mainly due to two reasons, (i) antimicrobial resistance genes are transferred from the environmental microbiota to various bacterial pathogens via various horizontal gene transfer mechanisms and (ii) the actual load of and identity of antibiotic resistance genes present in the natural environment is still not identified correctly.

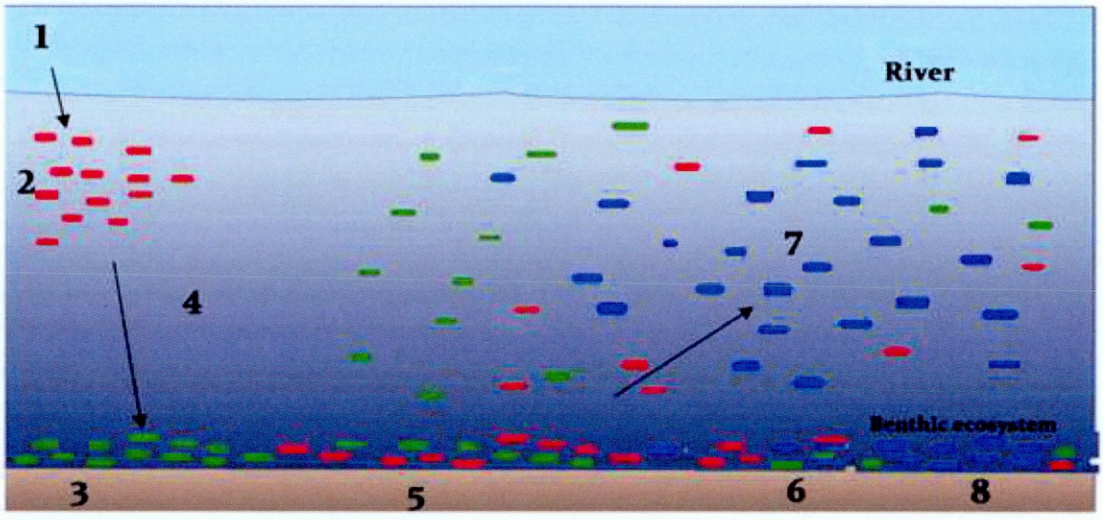

Figure 6.1: Schematic representation showing the role of benthic environment of river ecosystem in serving as reservoir for antibiotic resistant bacteria. Where; (1 & 2) Introduction of antibiotic resistant bacteria (red) by anthropogenic activity; (3) Indigenous non-pathogenic bacteria (green); (4) Sedimentation of antibiotic resistant bacteria; (5) Interaction between non-pathogenic bacteria and antibiotic resistant bacteria; (6) Acquisition of antibiotic resistant gene via horizontal gene transfer; (7) Dispersal of antibiotic resistant bacteria (blue) to the water column above the benthic level and (8) Novel antibiotic resistant bacteria.

This study adds to the large body of evidence that the environment, especially aquatic ecosystems, harbours multidrug resistant pathogens. In addition to this, confirmation of the existence of plasmid- and efflux pump-mediated resistance among Gram-negative pathogens isolated from the river is suggestive of the possibility of a rapid spread of drug resistance among

non-pathogenic indigenous bacteria, which in due course may reach humans. The present study also points out the role of environment in moulding pathogens to exhibit and express higher virulence when compared to their laboratory-raised counterparts. Therefore, more studies in this field are warranted in order to understand the complex succession of attainment of resistant genes by human pathogens (Martínez, 2012). Well planned, coordinated and proactive steps should also be taken at a global scale to counter the emergence and spread for multi-drug resistant pathogens with special emphasis on our natural ecosystems; e.g. water bodies. It is essential to enhance the surveillance / implement control measures and failure or further delay to do so may lead mankind to the inevitable beginning to "*antibiotic apocalypse*" (MacKenzie, 2015).

6.2 Suggestions for future work

The results of the present study prove that anthropogenically polluted river can act as a reservoir of antibiotic resistant bacteria. The role of seasonal variations in bacterial load and benthic environment of river ecosystem in housing antibiotic resistance bacteria remains to be clarified. Therefore recommendations for future work include:

1. Isolation and identification of bacterial isolates with multi-drug resistance from sediment samples of river environment.
2. Study of seasonal variations in bacterial load and diversity of antibiotic resistant bacteria in the sediment and water samples. This can be achieved by periodic sampling of water from the same site during different seasons. The results obtained will help us to study the role of season-dependant physical and chemical variations in survival of antibiotic resistant bacteria.
3. SDS-PAGE comparison of environmental and laboratory isolates exhibited marked variations in outer membrane proteins concentrations. This was more pronounced in case of outer membrane protein of *Enterobacter* spp. and *P. stutzeri.* Purification, characterization and comparison of such proteins may help us to better understand and molecular mechanism by which environmental strains attain antibiotic resistance. Purification of protein can be carried out using gel filtration chromatography or electro-elution. The intact mass and amino-acid sequencing of purified proteins can be done using MALDI-TOF mass spectroscopy and N terminal amino acid sequencing.

in silico analysis and protein modelling of virulence genes of *OprC* gene of *P. stutzeri* and *tetC* gene of *E. agglomerans* from environmental strains and laboratory strains. The comparison of

sequences and 3-D structures will help study the variation exhibited by environmental strains to achieve resistance.

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