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Antibiotic Resistance in Northern Mississippi Wetlands

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ANTIBIOTICS RESISTANCE PATTERN IN NORTHERN MISSISSIPPI WETLANDS

by
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ABSTRACT

Shama Moktan: Antibiotics Resistance Pattern in Northern Mississippi Wetlands
(Under the direction of Dr. Lydia Halda-Alija)

Bacterial resistance to antibacterial agents is a condition in which there is no susceptibility or decreased susceptibility to antibacterial agents that ordinarily cause cell death or inhibition of bacteria. Bacterial resistance to antibacterial agents is a quantitative measurement of the efficiency or concentration expressed in micrograms per milliliter or as inhibition zones in millimeters for the diffusion technique of an antibacterial agent against a specific bacterium. *In vitro* methods for measurement of antibacterial activity are available that are based on testing increased concentrations of antibacterial agent against a bacterial isolate to identify at which concentration the growth of the bacterium is inhibited. The lowest concentration at which detectable growth is inhibited is known as the minimum inhibitory concentration (MIC) of the antibacterial agent. Indeed the MIC indicates the relative measurement of the smallest concentration of antibacterial agent required to inhibit the growth (cell division) of a bacterium.

Bacterial isolates from clinical environment are regularly tested for antibiotics susceptibility. Clinical isolates are selected for antibiotic susceptibility testing to monitor antibiotic resistance and multidrug-resistance so as to enhance medical therapy. However, with the increasing use of antibiotics outside the clinical area, it has become necessary to determine the antibiotics susceptibility of environmental bacterial isolates. It has become important to determine the spread of resistant organisms throughout the environment.

Environmental isolates obtained from northern Mississippi wetlands were tested for antibiotic susceptibility to different class of antibiotics including novel antibiotics. Antibiotics susceptibility testing of the environmental isolates classified as being pathogenic were done using microdilution protocol specified by the National Committee on Clinical Laboratory Standards (NCCLS). The degree of growth response of each isolate in response to a particular antibiotic was recorded. Antibiotic potency for a particular isolate was determined in terms of the IC_{50} concentration (antibiotic concentration that affords only 50% growth of the bacteria), the minimum inhibitory concentration (lowest concentration of the antibiotic at which growth of bacteria is limited) and the minimum bactericidal concentration (the minimum concentration of the antibiotic at which growth of the organism is completely inhibited). MIC values of the antibiotics in the presence of each environmental isolate were compared to the MIC interpretative standards ((μg/ml) for Enterobacteriacae according to NCCLS. Data from different antibiotics susceptibility testing suggested that the environmental isolates were resistant to the earlier generation of β-lactam class of antibiotics. On the contrary, resistance to tetracycline, a commonly used antibiotic in agriculture, was found to be intermediate. The environmental isolates were generally susceptible to newer generation of β-lactams, such as cefoxitin and ceftazidime. Antibiotic resistance to β-lactams is mainly attributed to the production of β-lactamase enzyme by bacteria that hydrolyzes the β-lactam ring of the β-lactams and reduces the effect of β-lactams. The newer generations of β-lactams possess a bulkier structure around the β-lactam ring to resist the

action of β-lactamase enzyme. The results of this study suggest that environmental β-lactamase enzyme did not evolve to recognize a bulkier structure of the new generations of β-lactams antibiotics.

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INTRODUCTION

Antibiotics Resistance

The emergence of antibiotic resistance in bacterial populations is a concerning issue for clinicians, health workers and the general public. Antibiotic resistance confers bacteria the ability to resist the effect of the antibiotic. Antibiotic resistant bacterial infection becomes more difficult to treat because the bacteria causing the infection cannot be killed or reduced by traditional antibiotics. Traditional antibacterial therapy becomes more difficult because the selection of antibiotics for the treatment is narrowed.

Bacterial resistance to an antibacterial agent is best described and defined in relation to bacterial susceptibility to that antibacterial agent. Bacterial resistance to antibacterial agents is a condition in which there is no susceptibility or decreased susceptibility to antibacterial agents that ordinarily cause cell death or inhibition of bacteria. Bacterial resistance to antibacterial agents is a quantitative measurement of the efficiency or concentration expressed in micrograms per milliliter or as inhibition zones in millimeters for the diffusion technique of an antibacterial agent against a specific bacterium (Madigan et al. 2002). It is a property of bacteria to resist the effect of antibiotics.

Extrinsic factors such as the concentration of a given antibiotic, duration of exposure to the antibiotics and availability of a given carbon source may regulate the expression of antibiotic resistance towards the antibiotic (e.g. Martinez and Baquero 2000). *In vitro* and *in vivo* studies have indicated that increased fitness may not always be the result of antibiotic resistant mutation (Levy 1992). However, should an antibiotic

resistant mutant be selective over the wild-type counterpart, it suggests that the antibiotic resistant mutation has conferred increased fitness to the bacteria.

Bacterial resistance to antibiotics can be either natural (intrinsic) or acquired. Antibiotic resistance can be encoded by the bacterial chromosome or by extra-chromosomal entities called plasmids (Levy 1992). The resistance gene encoded in the chromosome is most likely a result of mutation within the chromosome which makes it an intrinsic property. Furthermore, the bacterial chromosome is stable and does not participate in horizontal gene transfer like the plasmid does. Therefore, antibiotic resistance genes encoded by the chromosome are usually not directly transmissible, at least horizontally. Antibiotic resistance can also be encoded in the plasmid level on so-called resistance plasmids (R factors) (Madigan et al. 2002). The resistance gene encoded in the plasmid, is most likely a result of acquisition of that gene from another bacteria. Therefore, antibiotic resistance through plasmid-encoded genes is an acquired property of the bacteria.

Plasmid-mediated transfer of antibiotic genes is possible through horizontal gene transfer. In the bacterial populations, horizontal gene transfer is carried out through the processes of transformation, conjugation and transduction. Transformation is simply the uptake of a "naked" DNA fragment by a bacterial cell, its further incorporation into the cell's genome or into plasmid and eventually, its expression in a chromosomal level or in a plasmid-level. Conjugation involves the transfer of plasmid DNA copy from a donor bacterial cell to a recipient cell. This mechanism requires direct cell to cell contact which is established through a special structure called pilus. Pili are seen only during the process of conjugation. The genetic material is transferred through this cell-cell bridge.

Transduction involves plasmid transfer via bacteriophages. When bacteriophages infect a bacterial cell, they exploit the bacterial genome for viral protein synthesis. The bacterial genome gets integrated with viral genome. After lysis and upon subsequent infections of other bacterial cells by these viruses, the previous bacterial genome gets transferred into other bacterial cells.

There are several mechanisms of antibiotic resistance in the bacterial population. As mentioned earlier, the genetic basis of these mechanisms could be either chromosomal or plasmid mediated or in some cases both.

Antibiotic resistance could be an intrinsic phenomenon for some bacteria due to the difference in cell features. For instance, a large portion of the bacterial population is either gram-negative or gram-positive. The difference between gram-negative and gram-positive bacteria is the cell wall (Figure 1). The gram-negative cell wall is a multilayered structure and quite complex, whereas the gram-positive cell wall consists primarily a single type of molecule and is often thicker (Madigan et al. 2002). Due to their protective outer membrane gram-negative bacteria are exclusively resistant to antibiotics that target cell wall synthesis inhibition, as opposed to gram-positive bacteria which are not. Some common antibiotics that target cell wall synthesis inhibition are ampicillin and penicillin. Although both penicillin and ampicillin belong to the β-lactam class of antibiotics, unlike penicillin, ampicillin is a broad spectrum antibiotic.

Figure 1. Gram-positive and gram-negative cell wall

Source: Available from URL: http://www.arches.uga.edu/~emilyd/theory.html

Figure 1.1: A Gram-positive cell wall

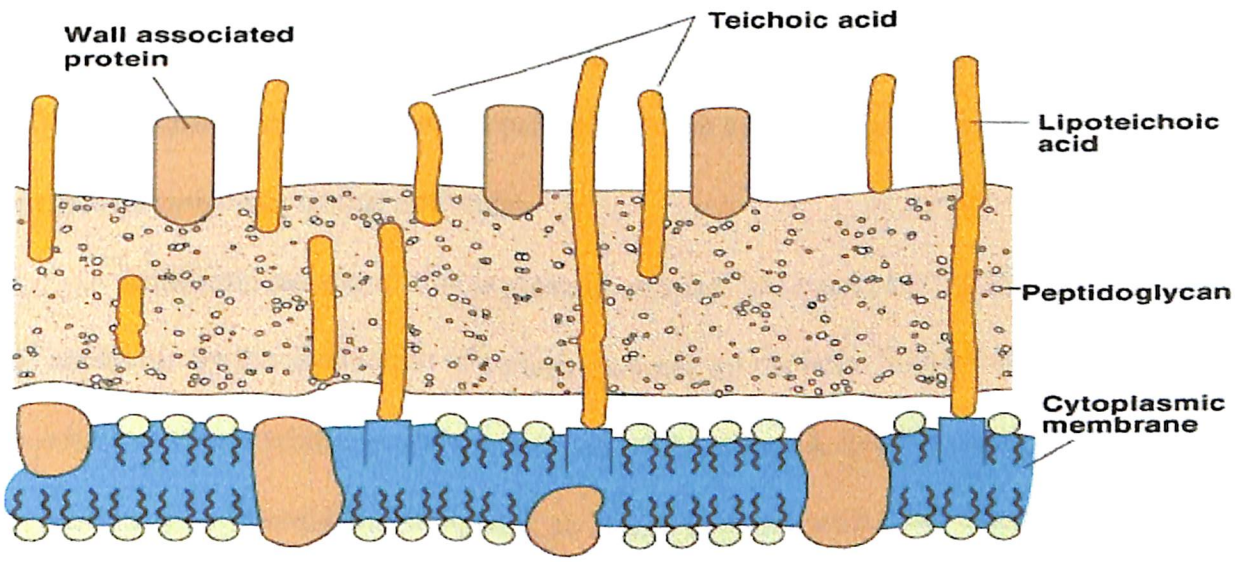
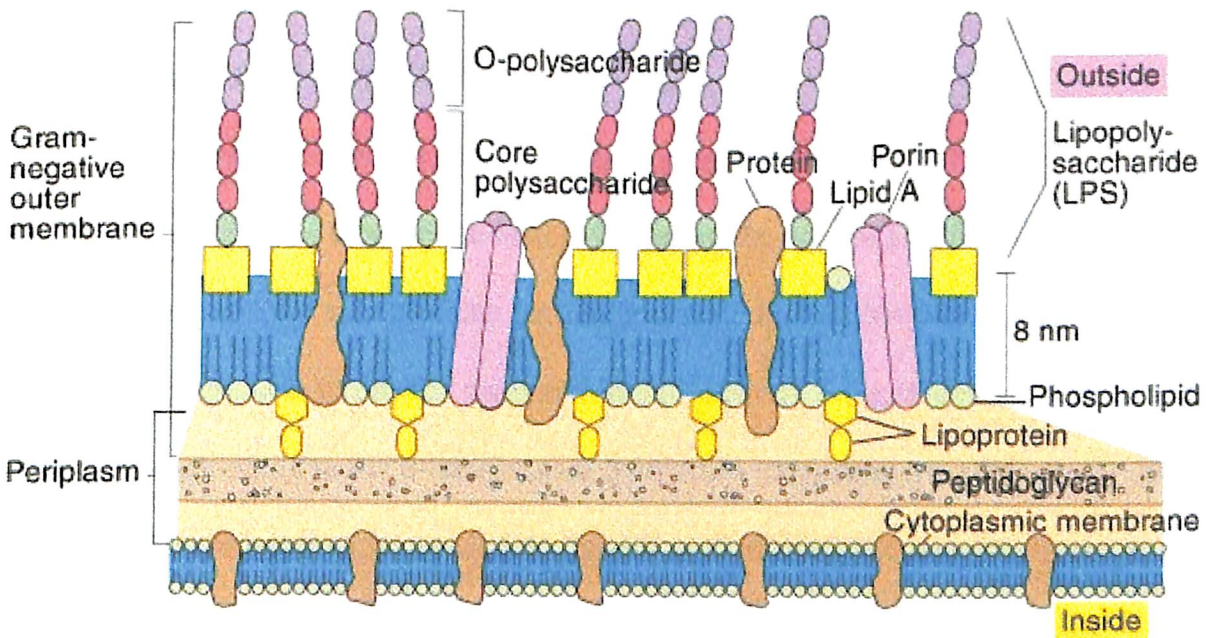


Figure 1.2: A Gram-negative cell wall



In addition some gram negative bacteria such as *Pseudomonas aeruginosa* and enteric bacteria have reduced permeability to penicillins (Madigan et al. 2002). Antibiotic resistance can also be achieved by altering the target site of the antibiotics. Studies have shown an alteration in DNA gyrase, a chief site of target of quinolones (Madigan et al. 2002). Alteration of a biochemical pathway that an antibiotic blocks can also confer antibiotic resistance to that antibiotic.

Antibiotic resistance that is plasmid encoded has been observed in the inactivation of antibiotic by the production of certain enzymes by bacteria. *Staphylococcus aureus* produce enzymes whose genes are encoded by the plasmid, that are capable of resistance towards aminoglycosides (Madigan et al. 2002). Plasmid mediated tetracycline resistance is also seen in enteric bacteria that are capable of tetracycline efflux through proteins that are plasmid encoded (Madigan et al. 2002). Bacteria capable of tetracycline efflux actively prevent the entry of tetracycline into the cell leading to decreased penetration of the drug into the cell (Levy 1984).

The result of antibiotic resistance through both chromosomal origin or plasmid mediation, in addition to selective factors including the type of antibiotic, allows bacteria to inhibit the mode of action of that particular antibiotic. In other words, the mutant bacteria are now able to impede the antibiotic's functions at the target site. For example, antibiotics belonging to the β-lactam class (penicillin, cephalosporin) (Figure 2) disrupt the formation of peptidoglycan layer in the cell wall of most bacteria. The result of which kills the bacteria by osmotic effect. However, bacteria possessing the β-lactamase gene produce the β-lactamase enzyme that is able to hydrolyze the β-lactam ring of β-lactam antibiotics. The bacteria which would initially have been susceptible to the β-lactam

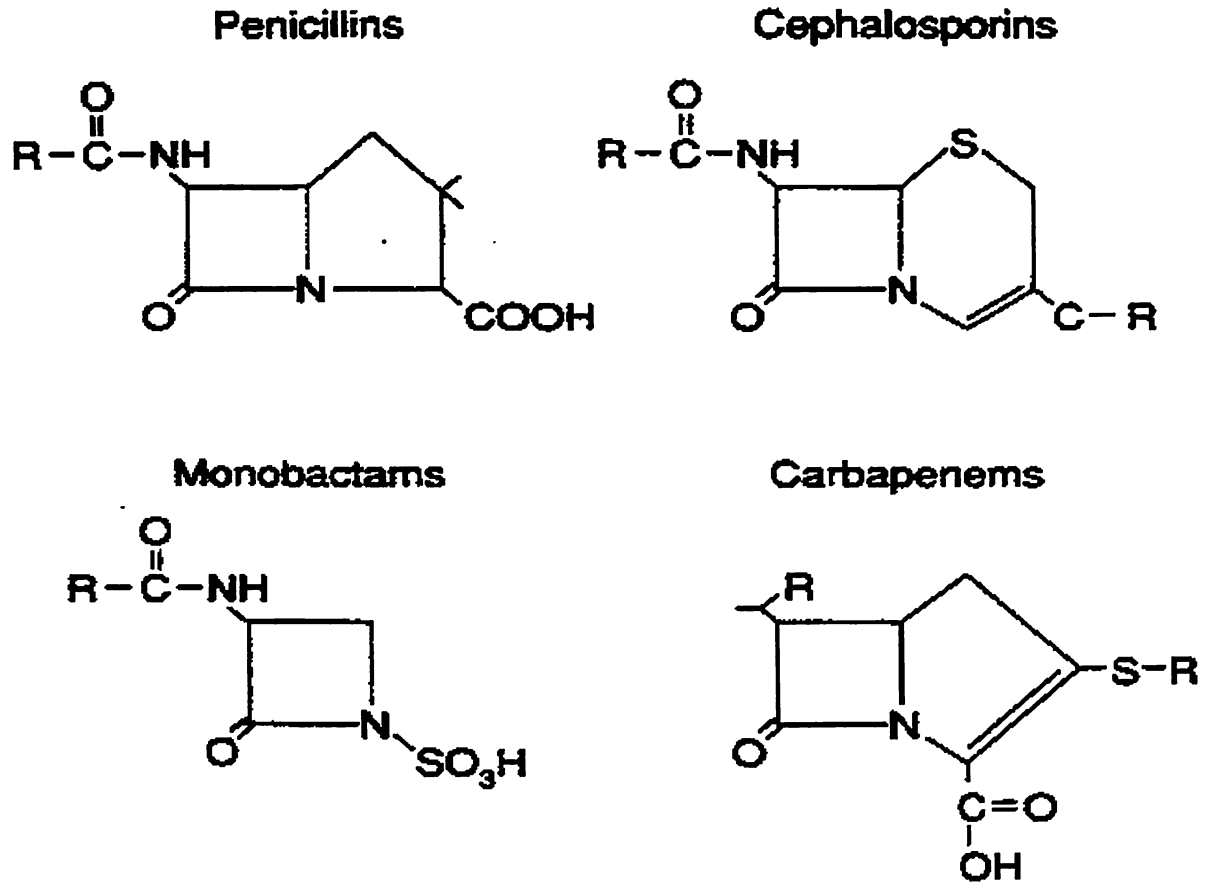
antibiotics are now resistant to its action. They are able to grow in the presence of these antibiotics.

Another antibiotic resistance related phenomenon has been multidrug resistance among various species of bacteria. Multidrug resistance among *Enterobacteriaceae* in the hospital setting is an increasing problem (Leverstein-van Hall, et al. 2002). In two separate studies conducted by Guerra, et al. and Leverstein-van Hall, et al. in 2001, spread and contribution of multidrug resistance was seen associated with plasmid mediation.

Emergence of antibiotic resistance patterns in bacteria in clinical settings have been extensively studied and documented. The appearance of antibiotic resistant pathogenic strains in nosocomial infection has become a trend. Vancomycin resistant *Enterococcus sp.* in nosocomial infections is one of them. In most cases, resistance to a new antibiotic arises within 3 years of the antibiotic's FDA approval date (Medieros 1997). This continuous victory by the bacteria over the antibiotics poses a serious threat to human health.

Figure 2. The β-lactam antbiotics possess a distinct ring in their chemical structure characterized as the β-lactam ring.

Source: Available from URL: http://www.lumen.luc.edu/lumen/MedEd/USMLE/antibiotic%20clases.ppt



Mechanism of action of different classes of antibiotics

There are a vast number of antibiotics that are commercially available. An antibiotic can be either bacteriostatic or bactericidal. A bacteriostatic antibiotic limits the growth of bacteria. A bactericidal antibiotic clears the bacterial population. The strength of an antibiotic is determined by its concentration at which 50% growth of the bacteria is inhibited (IC_{50}), its minimum inhibitory concentration (MIC) and its minimum bactericidal concentration (MBC). The minimum inhibitory concentration is the lowest concentration of the antibiotic which supports no visibly detectable growth of the bacteria. The minimum bactericidal concentration is the lowest concentration that kills 100% of the bacteria.

Antibiotics are classified under different classes on the basis of their mechanism and target (Table 1).

Table 1. Antibiotics used and their mechanisms of action
Source: Madigan et al. 2002

Antitibiotics	Mechanism of Action	Spectrum of activity	Class
Ampicilin[a,b,c], Benzylpenicillin[c], Carbenicillin[c], Cefoxitin[c], Ceftazidime[c], Cephalosporin C[b]	Inhibit transpeptidation step in peptidoglycan synthesis; bind penicillin-binding proteins, stimulate autolysins	Gram-positive and/or gram-negative bacteria (depends on agent)	β-Lactams
Vancomycin	Inhibits transglycosylation and trasnpeptidation steps in peptidoglycvan synthesis	Most effective against gram-positive bacteria	Glycopeptides
Kanamycin[a], Gentamycin[a], Streptomycin[a], Neomycin[a]	Binds to 30S subunit of bacterial ribosome	Broadly bactericidal	Aminoglycosides
Tetracycline[a,b]	Binds to 30S subunit of bacterial ribosome; disrupt bacterial membrane	Broadly bacteriostatic; some protozoa	Tetracyclines
Erythromycin[a,b]	Binds to 50S ribosomal subunit	Bacteriostatic for most; bactericidal for some gram-positive bacteria	Macrolides/lincos-amides
Ciprofloxacin[a]	Binds to DNA gyrase	Broadly bactericidal; can enter phagocytes, kill intracellular bacteria	Fluoroquinolones
Rifampicin[a]	Binds β-subunit of bacterial RNA-polymerase	Broadly antibacterial; effective against mycobacteria	Rifampin
Tazobactam[c]	Inhibits the action of β-Lactamase		β-Lactamase inhibitor
Aztreonam[c]	Inhibits peptidoglycan synthesis	Gram negative	Monobactam
Linezolid[a]	Binds to 50S ribosomal subunit; interfere in protein synthesis initiation	Gram-positive	Oxazolidinones

[a] used in first antibiotic susceptibility testing
[b] used in second antibiotic susceptibility testing
[c] used in third antibiotic susceptibility testing

β-Lactams are types of antibiotics that target the penicillin-binding-proteins (PBP) in the bacterial cell wall. PBP are enzymes that provide peptidoglycan linkages within the cell wall. By targeting this crucial protein needed for the synthesis of bacterial cell wall, β-lactams actively stimulate autolysins in bacteria (Levy 1992). Some widely used β-Lactams are ampicillin, penicillin and cephalosporins.

Presumably, the use and overuse of β-lactams over the years has applied selective pressures on the bacterial population to counteract the effect of β-lactams. The emergence of the β-lactamase gene in gram negative bacilli was seen in the early 1960s. β-lactamases hydrolyze the amide-bond in the β-lactam ring. This reduces the function of β-lactams. The most notable β-lactamases are the TEM-1 and SHV-1 (sulfahydryl variant) and their variants. TEM-1 and SHV-1 preferentially hydrolyze the β-lactam ring of penicillin. However, variants of these β-lactamases can arise from single point mutation in the genes that encode these enzymes. The variants arising from such mutations are capable of acting on an extended-spectrum of β-lactam antibiotics and are called extended-spectrum β-lactamase (ESBL) (Bradford 2001).

The gene encoding the TEM-1 β-lactamase is plasmid and transposon mediated which accounts for the wide-spread occurrence of this particular enzyme in the bacterial population. It is found in many different species of members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Neisseria gonorrhoea*. Up to 90% of ampicillin resistance in *E.coli* is due to the production of TEM-1 (Bradford 2001). TEM-3 was the first β-lactamase identified to express an extended-spectrum-β-lactam (ESBL) phenotype. There are over 25 different variants of SHV that have been described. SHV-1 β-lactamase is usually plasmid mediated in *Escherichia coli* (Bradford 2001).

Clinically significant and nonrepeat isolates of *E. coli* and *Klebsiella sp* sampled for a period of 1 year in 12 participating Canadian tertiary care hospitals have been found to produce ESBL belonging to Amber's molecular class A (Mulvey, et al. 2004). Although Mulvey et al., indicate that the number of ESBL producers in Canada are relatively lower than other countries (approximately 5 to 15% of *Klebsiella sp.* and 2 to 10% of *E. coli* strains harboring ESBL(s) have been surveyed in U. S hospitals), their results point to the an alarming concern that ESBL producers have already gained a foothold in the bacterial pool associated with nosocomial infections.

This is where the use of β-lactamase inhibitor in tandem with β-lactam comes into play for the treatment β-lactam resistant infections. β-lactam/β-lactamase inhibitor combinations in treatment of various infections produced at least as efficacious, if not superior results compared to conventional regimen of antibiotics (Lee, et al. 2003).

In vitro combination of the third generation cephalosporin, Ceftazidime and the β-lactamase inhibitor Sulbactam decreased the growth of ESBL producing *Klebsiella pneumoniae* and *E. coli* (Lavigne, et al. 2004).

Antibiotic Resistance in Non-Clinical Setting

Another arena where appearance of antibiotic resistant bacteria has also been observed is in the environment. The extensive use of antibiotics in the animal feed, agriculture, etc. can provide easy route for antibiotics to leak into the environment and contribute to the selection of antibiotic resistant bacterial populations there. This situation is further enhanced by the non-medical use of antibiotics as a prophylactic and growth enhancement measures in livestock and domestic animals feeds as well. The discovery that antibiotics can improve the growth rate of animals, in the early 1950s, on the basis of yet unknown mechanisms, led to the massive use of these drugs as food additives (Amábile-Cuevas 1993) in animal feed.

Increased introduction of antimicrobial agents into the environment via medical therapy, agriculture, and animal husbandry has resulted in new selective pressures on bacterial populations (Col and O'Conner 1987). In the United States alone, 40,000 – 50,000 pounds of antibiotics are used each year just for the control of bacterial infections of fruit trees (Levy 1992). Wide spread usage of antimicrobial agents cause strain on bacteria that are constantly exposed to these agents leading to selection of resistant bacteria. Resistance to antibiotics becomes a necessity for the growth and survival of bacteria.

The use of antibiotics is not restricted to treatment of clinical diseases due to bacterial infection(s) in humans and animals. Antibiotics are constantly used for controlling vegetable and fruit infections as well. For example, streptomycin and oxytetracycline are used for fire blight and bacterial spots in plants, respectively (Levy 1992). In addition, antibiotics such as oxytetracycline, sulfamerazine are used to treat

ulcers or furunculoses of the skin of the fish (Levy 1992). Additionally, tetracycline is also widely used in the catfish and the salmon industry for treatment of infections. Especially since salmon farming requires placing the salmon pen in natural sea waters, antibiotics resistant strains of bacteria created by these farms will have contact with other marine life (Levy 1992). Consequently this situation poses a selective force for producing more resistant bacterial strains in yet another environmental niche (Levy 1992). Incidentally, tetracycline is added in animal feed for treatment of disease, prophylactic use and as a growth promoter. In a different study conducted by Chee-Stanford, et al. (2001) isolates from groundwater samples and lagoons used for swine waste disposal were analyzed. The researchers identified the tetracycline resistance gene in isolates from groundwater and lagoons associated with swine farms.

The isolation of resident stream species from a river downstream of a wastewater treatment plant discharge from the Arga River in Spain recovered enterobacteria and *Aeromonas* strains. These isolates showed increased resistance to several antibiotics including nalidixic acid, tetracycline, β-lactam, and co-trimoxazole. The percentage of acquired resistance for enterobacteria was less than for the *Aeromonas* strains (Goñi-Urriza, et al. 2000). Researchers project that this could be due to the resistance bacteria that are passing through the treatment process and conferring resistance to native bacteria (McArthur and Tuckfield 2000).

Recent evidence also suggests that heavy metal concentration (Mercury) in the sediments may be the strongest predictor of antibiotic resistance (McArthur and Tuckfield 2000). Bacterial resistance to streptomycin and kanamycin were positively correlated with sediment mercury concentration in streams below nuclear reactors and

industrial facilities, a result of indirect selection of metal tolerance (McArthur and Tuckfield 2000).

A class of antibiotics known as quinolones is widely used in veterinary medicine, particularly in Europe. These antibiotics, however, are excreted as unchanged substances and are among the most persistent drugs in the environment (Goñi-Urriza, et al. 2002). Quinolone resistance in environmental isolates from two European rivers (the Arga River in Spain and the Garonne River in France) has been identified. Quinolone resistance is due to the alteration of the target enzymes, the type II bacterial topoisomerases (Goñi-Urriza, et al. 2002).

Resistant bacteria selected in animals and plants may not directly cause diseases in humans. However, it is likely that they contribute their resistance genes into the environmental pool. Thereafter, horizontal gene transfer can enable the transfer of the resistance genes to bacteria that do cause diseases in humans.

Why study bacterial isolates from non-clinical environment?

It is important to study bacterial isolates from non-clinical environment and not just clinical environment for antibiotic resistance in order to monitor the spread of antibiotic resistance or even multidrug resistance in the environment. Effective compilation of the antibiotic patterns can be used as a useful environmental indicator of contamination in the environment due to direct or indirect anthropogenic effects.

Bacterial isolates for this study were collected from wetland environment (Halda-Alija 2003). Antibiotic resistance pattern of these environmental isolates can be established using National Center for Clinical Laboratory Standards (NCCLS) designed protocols on antibiotic susceptibility testing.

Previous data have shown that monitoring the response of antibiotic resistant enteric bacteria, rather than the entire assemblage, is a potentially productive approach to the examination of the responses of natural populations of bacteria to anthropogenic disturbances (e.g. Halda-Alija and Subgani 2004; Halda-Alija, et al. 2000). In previous studies, bacteria samples were obtained from sediment samples from pristine environment, wetland sediments and the rhizosphere of *Juncus effusus* L (Halda-Alija and Subgani 2004). The *Enterobacter* spp., *Aeromonas* spp., *Pseudomonas* spp., and *Bacillus* spp., were isolated. Bacterial samples from the rhizosphere were also included because genetic exchange and transfer of antibiotic resistance genes may be enhanced in the rhizosphere (Halda-Alija 2003). Bacterial isolates were subjected to antibiotics susceptibility testing with different antibiotics with different mechanisms of action (Table 1). Antibiotic susceptibility was determined by the plate agar and disk diffusion tests. The rate of acquired ampicillin resistance (<50μg/ml) was high for ampicillin for 98 out of

137 (72%) of the bacteria tested (Halda-Alija and Subgani 2004). The rate of resistance to ampicillin suggests acquired resistance to ampicillin (Halda-Alija and Subgani 2004). The rate of acquired resistance was low (>10 to 20 μg/ml) for kanamycin, tetracycline and chloramphenicol for all strains tested. These preliminary findings suggested that the isolates are particularly resistant to β-Lactams.

Escherichia coli is considered the most widely studied species of bacteria, and the family Enterobacteriacae as a whole is the best studied group of microorganisms (Halda-Alija 2001). Enterobacteriacae are ubiquitous. They are distributed worldwide and are found in water and soil (Halda-Alija et al. 2000; Halda-Alija et al. 2001) and as normal intestinal flora in humans and many animals (Grimont and Grimont 1992). They are responsible for a wide array of human diseases but were rarely reported as pathogens before widespread use of antibiotics (Schaechter et al. 1999). Conjugation and associated transfer of antibiotic resistance genes is most readily demonstrated in *E. coli* and related members of Enterobacteriacae (Dale 1998). Therefore, we want to assess the antibiotics susceptibility of the enteric bacteria isolates obtained from the aforementioned non-clinical environment.

The enteric bacteria isolates were subjected to antibiotics belonging to different class and having different spectrum of activity (Table 1) for antibiotic susceptibility testing. The preliminary data suggest that the bacterial isolates were particularly resistant to antibiotics belonging to the β-lactam class (Halda-Alija and Subgani 2004). Out of the bacterial strains that were tested during these preliminary studies, 12 strains were selected for further susceptibility testing against antibiotics belonging to common chemical classes (penicillins, cephalosporins, fluoroquinoloes, tetracyclines and macrolides) using

NCCLS protocol on antibiotic susceptibility testing. A novel class of antibiotic, oxazolidinones was also included in this susceptibility testing. In addition chloramphenicol was included in this antibiotic susceptibility testing. Chloramphenicol is primarily bacteriostatic and inhibits bacterial protein synthesis by binding to the 50s subunit of bacterial ribosome. It has a wide spectrum activity against gram-positive and gram-negative bacteria.

A modified version of the broth microdilution protocol designed by the National Center for Clinical Laboratory Standards (NCCLS M100-S12 2002; NCCLS M7-A5 2000) was employed for antibiotic susceptibility testing of the environmental isolates. NCCLS laboratory guidelines and procedures are standardized consensus through participation by individual laboratories, laboratory professional associations, industries and government agencies. Therefore, NCCLS standards represent selective criteria for effective outcome (NCCLS M7-A5 2000).

Antibiotic susceptibility testing of the environmental isolates was also performed with different generations of β-lactams and β-lactamase inhibitors. This was done to distinguish any difference in responses that these strains might have towards newer antibiotics.

METHODS AND MATERIALS

In vitro methods for measurement of antibacterial activity are available that are based on testing increased concentrations of antibacterial agent against a bacterial isolate to identify at which concentration the growth of the bacterium is inhibited. Conventional methods of antibiotics susceptibility assessment include disk diffusion technique in which the zone of inhibition is measured in millimeters. The diameter of the zone of inhibition can be used to identify whether a bacterial strain is resistant or susceptible to an antibiotic. The results of conventional methods such as the disk diffusion technique for antibiotic susceptibility are generally verified by other methods as well. Alternative methods are adapted in order to confirm the selective bacterial populations that are resistant to antibiotics. The microdilution procedure for antibiotics susceptibility testing used in this study is selected for its high throughput screening capacity. It is based on NCCLS protocol for dilution antimicrobial susceptibility tests for bacteria that grow aerobically (NCCLS M7-A5 2000).

Bacteria strains:

Bacterial isolates were identified and categorized. Following bacterial strains have been used in this study:

FD1	132	48	J2	67	124
F18	F5	F7	FD2	F1	D3

Quality Control Strains:

Escherichia coli ATCC® 25922, *Escherichia coli* ATCC® 35218 and *Enterobacteria cloacae* ATCC® 13047 strains from American Type Culture Collection,

Rockville, MD were used as reference strains as per quality control guidelines set by NCCLS (NCCLS M7-A5 2000).

Short-term storage of the bacteria and the quality control strains:

Bacterial strains were streaked on Eugon agar (Difco, Detroit, MI) Petri plates. The agar plates were incubated at 37°C for 24 hours. The plates were stored at 4°C for future use.

Antibiotics storage:

All antibiotics in this study were dissolved in Dimethyl Sulfoxide (DMSO), except for gentamycin, neomycin, kanamycin and streptomycin that were dissolved in nanopure water. The antibiotics were dissolved to a concentration of 5.12 mg/ml. Aliquots (80μl) of antibiotic solutions were stored in the first column of a 96 well flat bottomed microplates (Costar) at -70°C.

Antibiotics Dilution:

On the day of the assay, the microplate containing the antibiotic solutions is removed from the freezer and allowed to thaw. The antibiotics in column #1 are diluted five times in sterile 0.9% saline. Then they are serially diluted three-fold until column # 11. Column #12, except the last two wells, contains only DMSO. DMSO is also diluted five times in 0.9% saline and represents the negative control of the assay. Thus, the concentration of the antibiotic samples in the dilution plate is between 1.024 mg/ml and 5.202×10^{-5} mg/ml. The diluted antibiotics are transferred in duplicates to a new microplate.

Preparation of bacterial inocula:

On the day of the assay, 1-2 colonies of the strain were transferred from the Eugon agar plates to sterile 0.9 % 3 ml saline. 0.5 McFarland suspension (5μl of 48mM $BaCl_2$ + 995μl of 180mM H_2SO_4) was prepared (NCCLS M7-A5 2000). The bacterial suspensions in saline and the 0.5 McFarland suspension were agitated on a vortex mixer to ensure uniform turbidity. Thereafter, 100 μl from each bacterial suspension, 0.5 McFarland suspension, 0.9% saline and 180mM H_2SO_4 were transferred in duplicates to a new 96 well microplate. The absorptions of the saline suspension were compared to that of 0.5 McFarland suspension at 630nm using the El-340 Biokinetics Reader (Bio-Tek Instruments, Vermont). The result of which was used to evaluate the inoculum size of enteric bacteria in Mueller-Hinton broth (at pH 7.3) to achieve a final target inoculum of 5.0×10^5 Colony Forming Unit/ml (NCCLS M7-A5 2000) after addition to the antibiotics. Normally, turbidity of 0.5 McFarland standard is comparable to turbidity of a cell suspension with 1.5×10^8 CFU/ml. Therefore, turbidity of bacterial suspension was calculated using the following expression:

$$\text{Turbidity of bacterial suspension} = \frac{\text{average (bacteria – saline)} \times 1.5 \times 10^8 \text{ CFU/ml}}{\text{average (0.5-}H_2SO_4\text{)}}$$

The inoculum size that needs to be added to the media to achieve a final target inoculum of 5.0×10^5 CFU/ml is calculated after multiplying the turbidity of each bacterial suspension with the following number:

$$20 \text{ ml} * \text{Number of plates} * 5 \times 10^5 \text{ CFU/ml} \times 1000 \text{ } \mu\text{l/ml} * (200/175)$$

Antibacterial Assay:

Finally, the inoculum calculated was added to the required volume of cation-adjusted Mueller-Hinton broth (at pH 7.3). Then 175.0 μl of the inoculum was pipetted to the diluted antibiotic samples to achieve a final volume of 200 μl. The final test concentration range of the antibiotic samples was now between 128 μg/ml and 0.002μg/ml. A 0 hour reading at 630nm of all the plates was taken.

The microtiter plates were incubated at 37°C for 24 hours. After the incubation period was over, each plate was sealed with SealPlate (Sigma-Aldrich, St. Louis, MO) and gently shaken to ensure that all the cells are in suspension. Then a 24 hour reading was taken for all plates at 630nm.

The antimicrobial assay is based on the difference of optical density readings of a particular strain at 0 hour and at 24 hour in the presence of antibiotic samples. The difference in the optical readings corresponds to the growth or lack of growth of that strain in response to the antibiotic samples. The antimicrobial assay used in this study allows to quantify the growth of a particular bacterial strain in response to serial concentrations of a specific antibiotic. It uses bacterial inoculum with Dimethyl Sulfoxide (DMSO) as the negative control (because DMSO should not inhibit growth). The last two wells of the last column of the 96 flat-bottom microtiter plate are designated for media (200 μl) only. The media represents the blank and no growth is anticipated in these two wells.

Any effect due to blank is subtracted out from the temporal difference of optical densities of a bacterial strain in response to the serial concentrations of a particular antibiotic. This gives the net growth of the bacterial strain in response to a particular antibiotic. The net growth is then compared to the average of the optical densities of the

negative controls. The ratio between the net growth of the bacterial strain in the presence of antibiotic and in the absence of antibiotic (negative controls) is multiplied with 100% to give % growth of the bacterial strain.

IC_{50}, MIC and MBC Determination:

These % growth values of each bacterial strain were plotted against serial dilutions of the antibiotic. The x-axis was presented in the log scale to encompass a wider range of concentration values in a small space. In a log scale the spacing between two data points allows for adequate placement to represent the serial dilution range of the antibiotics used in the assay. It also enables a better viewing of points of interception. The % growth values or the growth curve was made to intercept the x-axis at 50%. Therefore, the point of interception of the growth curve on the x-axis was recorded as the IC_{50} value of that antibiotic (Figures 3-9).

The MIC was determined by observing the microtiter plate and selecting the well at which there was no visible sign of growth of the bacteria. The corresponding concentration of that well was determined to be the MIC of that antibiotic.

To determine the MBC, 5 μl from each well that showed no apparent signs of growth were transferred to Nutrient agar (Difco) Petri plates. The agar plates were incubated at 37°C for 20 - 24 hours. The lowest concentration that did not allow growth or recovery of the organism on the agar plate was selected to be MBC.

Standard deviation and average of IC_{50}, MIC and MBC values from replicate studies of each antibiotic was determined using Microsoft Excel 2002. MIC and MBC values of the second antibiotics susceptibility testing and the β-lactam antibiotics susceptibility testing were graphed using GraphPad Prism (Figure 10 and 11).

Figure 3. Growth curves of bacterial strain J2

Figure 3.1: Growth curves of J2 showing IC_{50}s for Erythromycin, Ampicillin and Tetracycline at 15.0, 3.5, and 0.15 µg/ml, respectively from the general antibiotics susceptibility testing

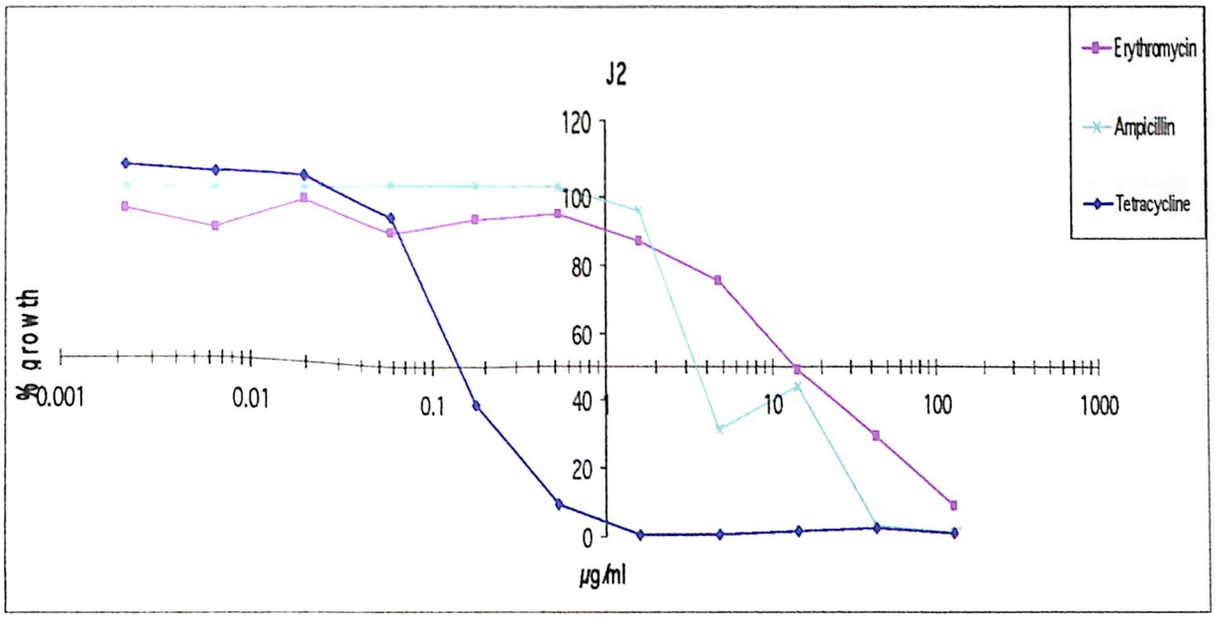
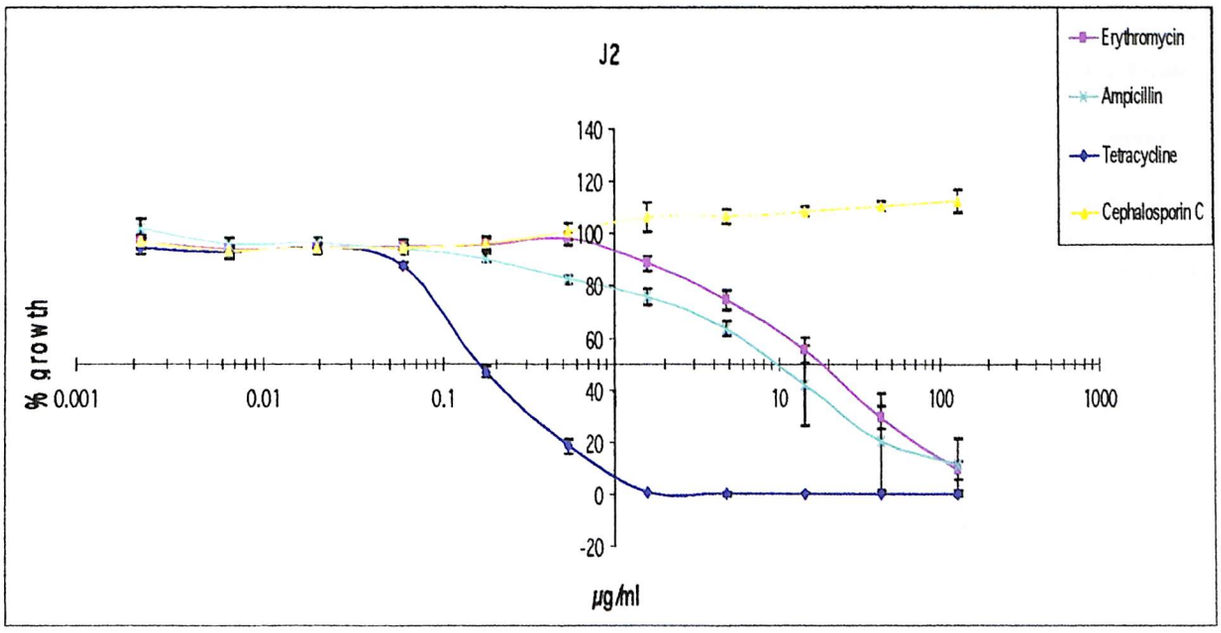


Figure 3.2: Growth curves[a] of J2 showing IC_{50}s for Erythromycin, Ampicillin and Tetracycline at 20.0, 9.5 and 0.15 µg/ml, respectively. Growth curve[a] against Cephalosporin C shows no inhibition by Cephalosporin C.



[a] average of three replicates

Figure 4. Growth curves of bacterial strain F1

Figure 4.1: Growth curves of F1 showing IC_{50}s for Erythromycin, Ampicillin and Tetracycline at 15.0, 15.0, and 0.35 µg/ml, respectively from the general antibiotics susceptibility testing

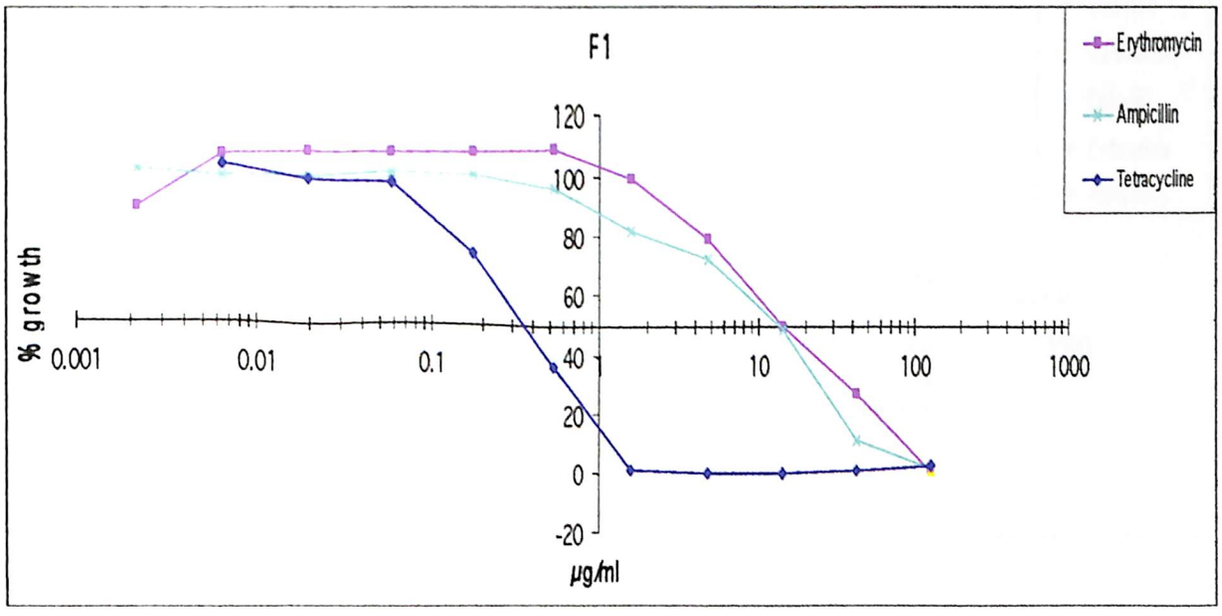
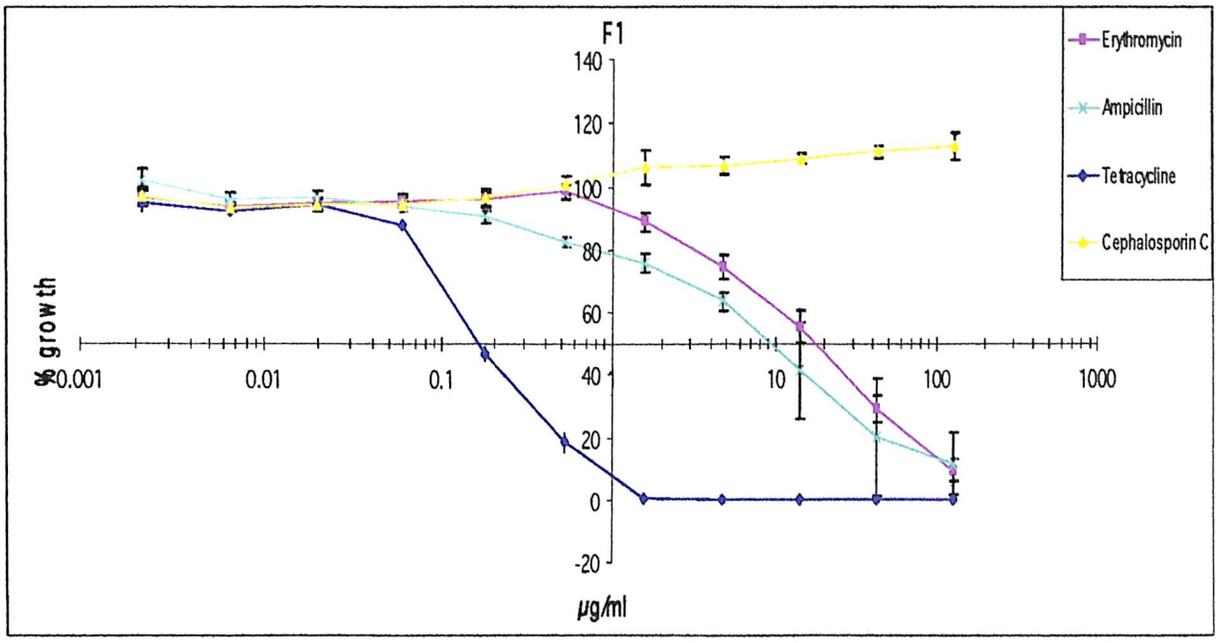


Figure 4.2: Growth curves[a] of F1 showing IC_{50}s for Erythromycin, Ampicillin and Tetracycline at 20.0, 9.5 and 0.15 µg/ml, respectively. Growth curve[a] against Cephalosporin C shows no inhibition by Cephalosporin C.



[a] average of three replicates

Figure 4.3: Growth curves F1 showing IC_{50}s for Ampicillin, Carbenicillin, Cefoxitin, Ceftazidime and Aztreonam at 6.0, 1.5 0.059, 0.065 and 0.02 µg/ml, respectively. Growth curve against Benzylpenicillin shows no inhibition by Benzylpenicillin.

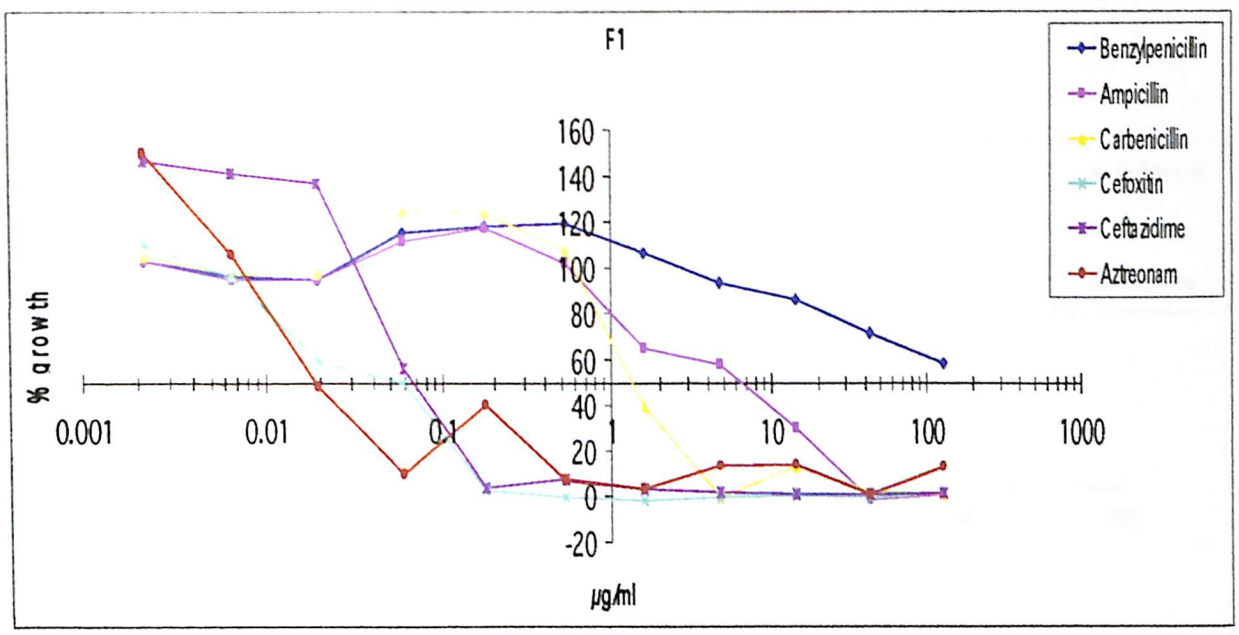


Figure 5. Growth curves of bacterial strain F18

Figure 4.1: Growth curves of F18 showing IC_{50}s for Erythromycin, Ampicillin and Tetracycline at 15.0, 20.0, and 0.40 µg/ml, respectively from the general antibiotics susceptibility testing

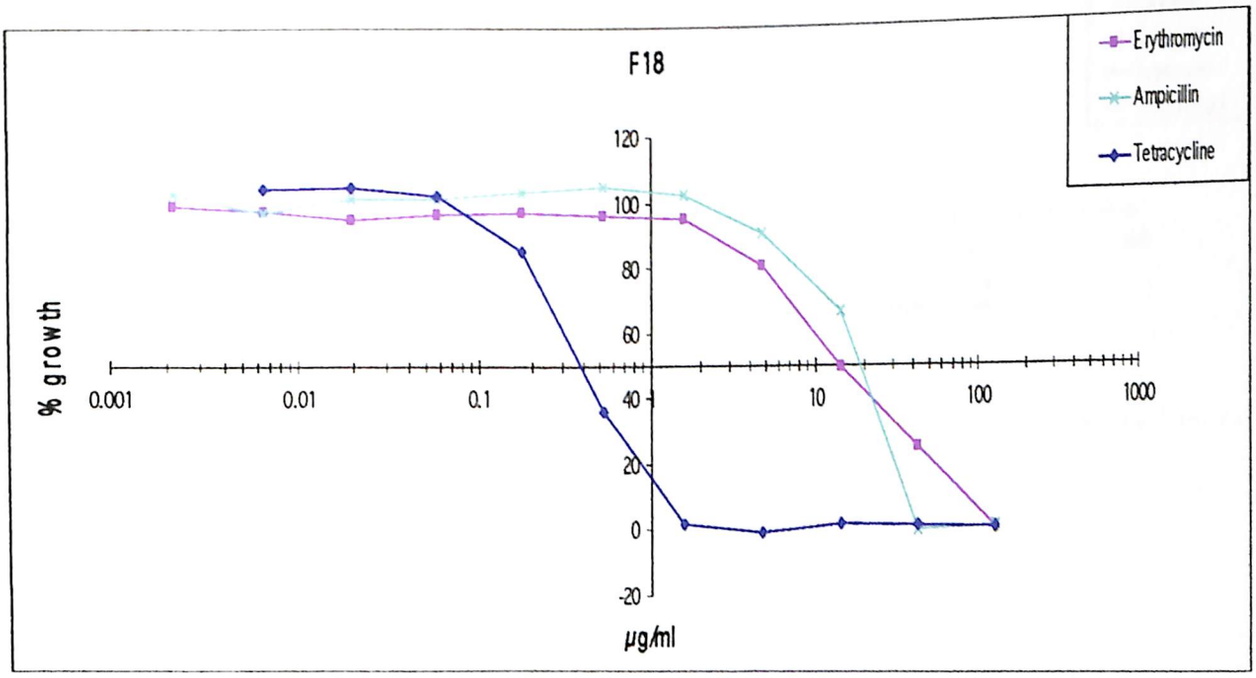
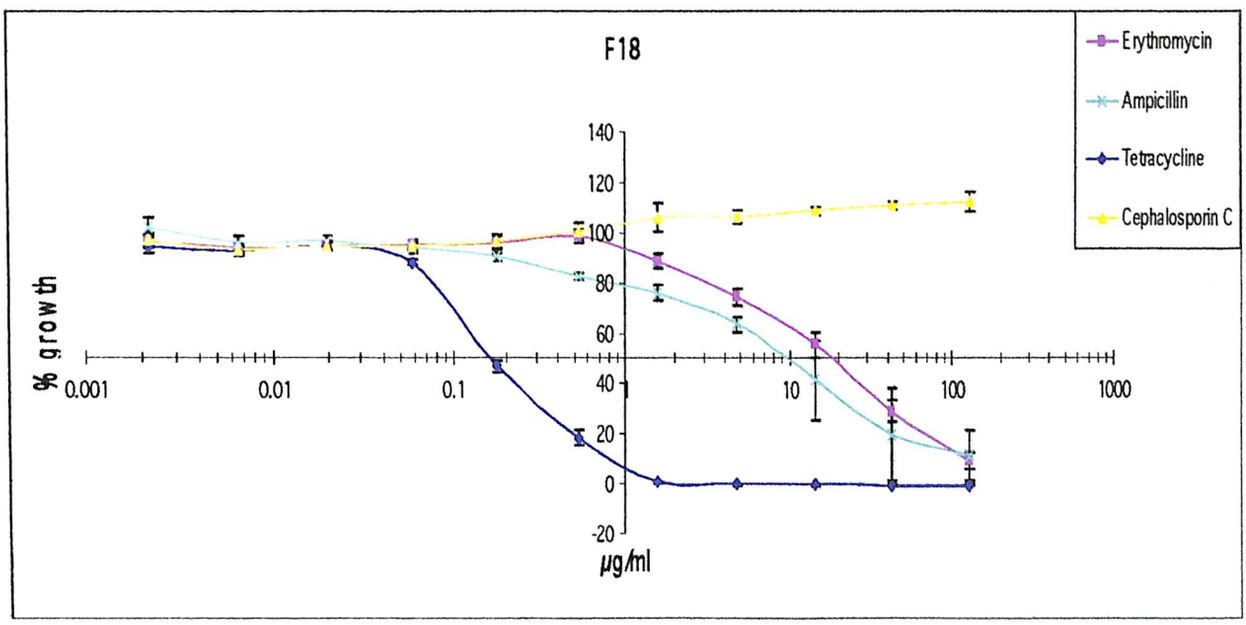


Figure 5.2: Growth curves[a] of F18 showing IC_{50}s for Erythromycin, Ampicillin and Tetracycline at 20.0, 9.5, and 0.15 µg/ml, respectively. Growth curve[a] against Cephalosporin C shows no inhibition by Cephalosporin C.



[a] average of three replicates

Figure 5.3: Growth curves of F18 showing IC_{50}s for Benzylpenicillin, Ampicillin, Carbenicillin, Cefoxitin, Ceftazidime and Aztreonam and Tazobactam at 80.0, 8.0, 1.5, 0.03 and 0.04, 0.015 and 75.00 µg/ml, respectively.

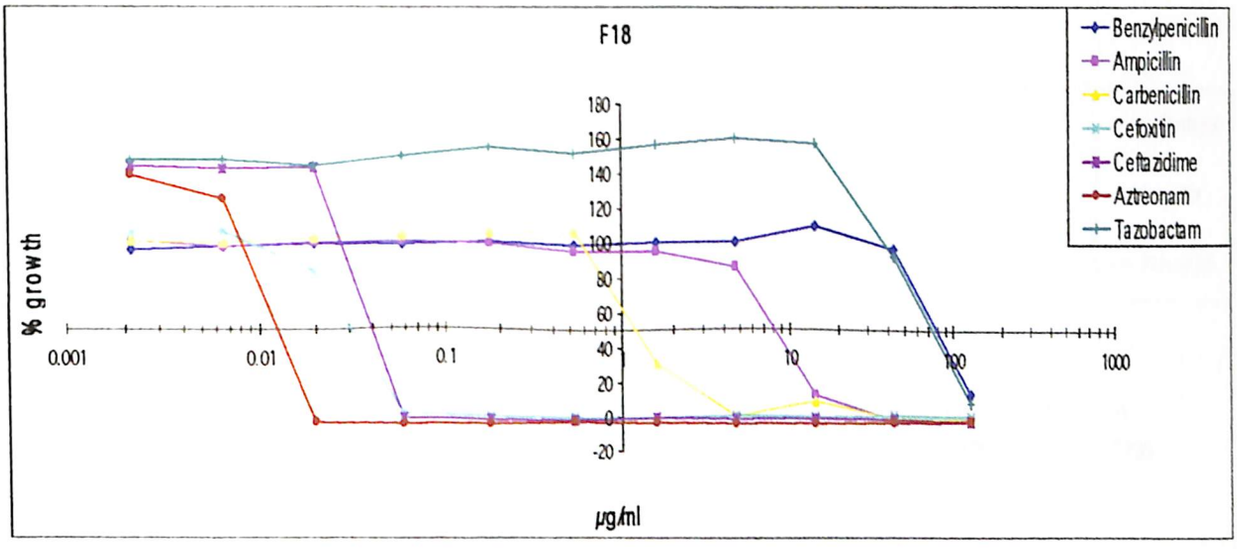


Figure 6. Growth curves of bacterial strain 48

Figure 6.1: Growth curves of 48 showing IC_{50}s for Erythromycin, Ampicillin and Tetracycline at 8.0, 4.5, and 0.30 µg/ml, respectively from the general antibiotics susceptibility testing

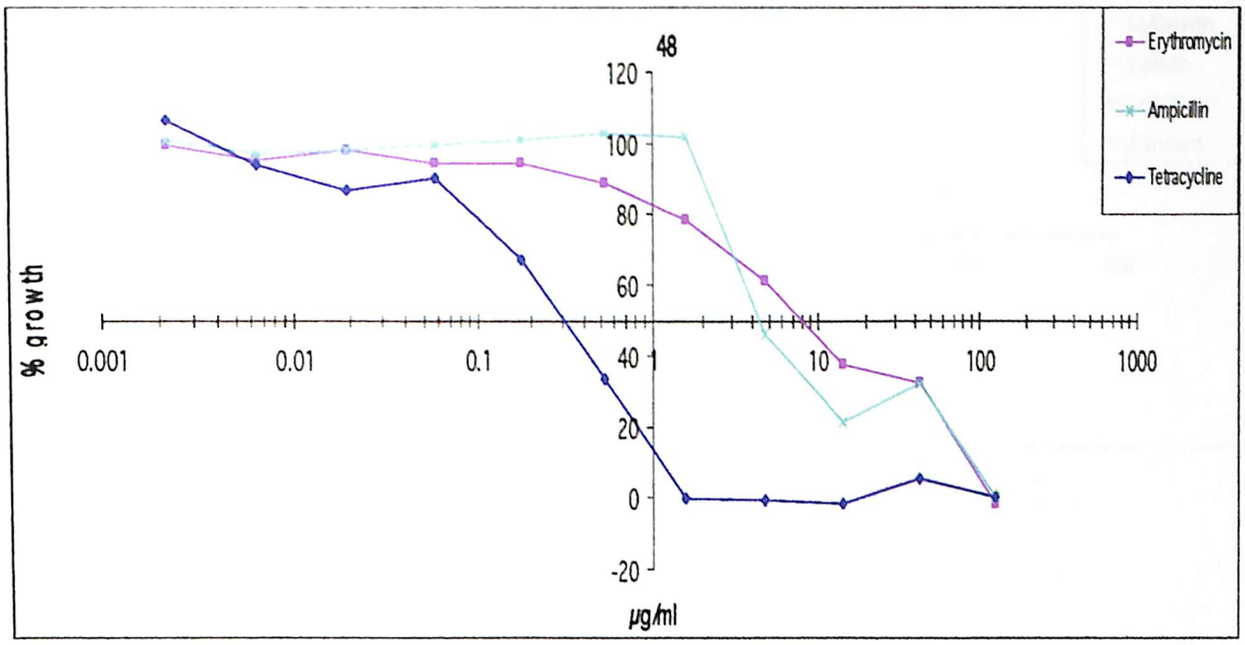
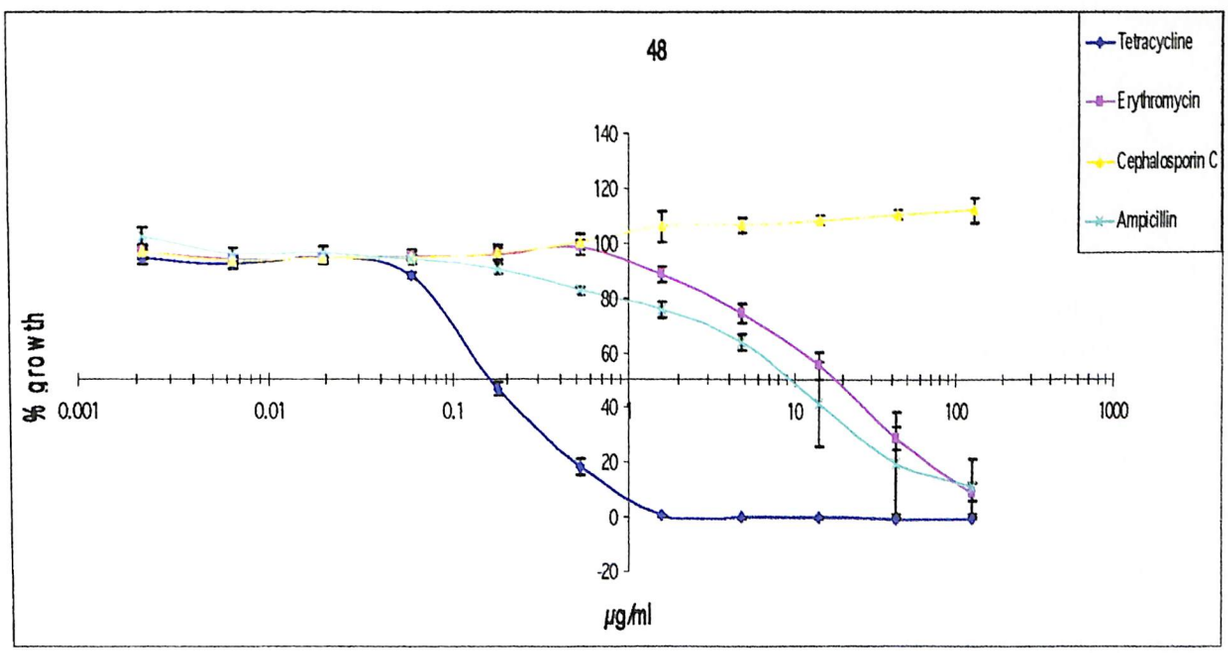


Figure 6.2: Growth curves[a] of 48 showing IC_{50}s for Erythromycin, Ampicillin and Tetracycline at 20.0, 9.5, and 0.15 µg/ml, respectively. Growth curve[a] against Cephalosporin C shows no inhibition by Cephalosporin C.



[a] average of three replicates

Figure 6.3: Growth curves of 48 showing IC_{50}s for Benzylpenicillin, Ampicillin, Carbenicillin, Cefoxitin, Ceftazidime and Aztreonam at 128.0, 60.0, 8, 0.025, 0.075, 0.15 and 0.03 µg/ml, respectively.

.

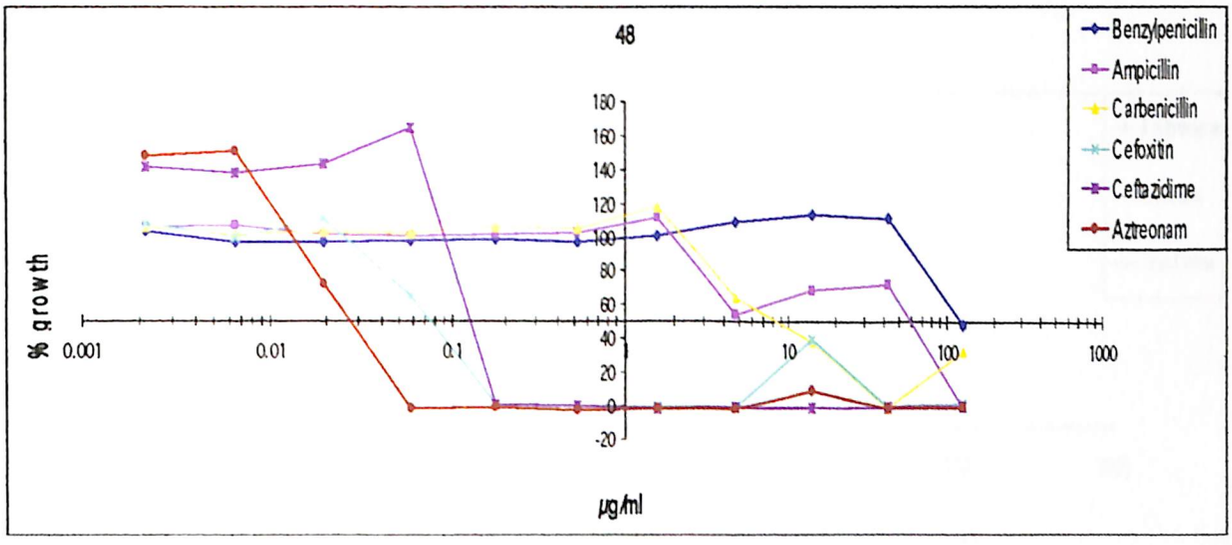


Figure 7. Growth curves of bacterial strain 67

Figure 7.1: Growth curves of 67 showing IC_{50}s for Erythromycin, Ampicillin and Tetracycline at 15.0, 20.0, and 0.40 µg/ml, respectively from the general antibiotics susceptibility testing

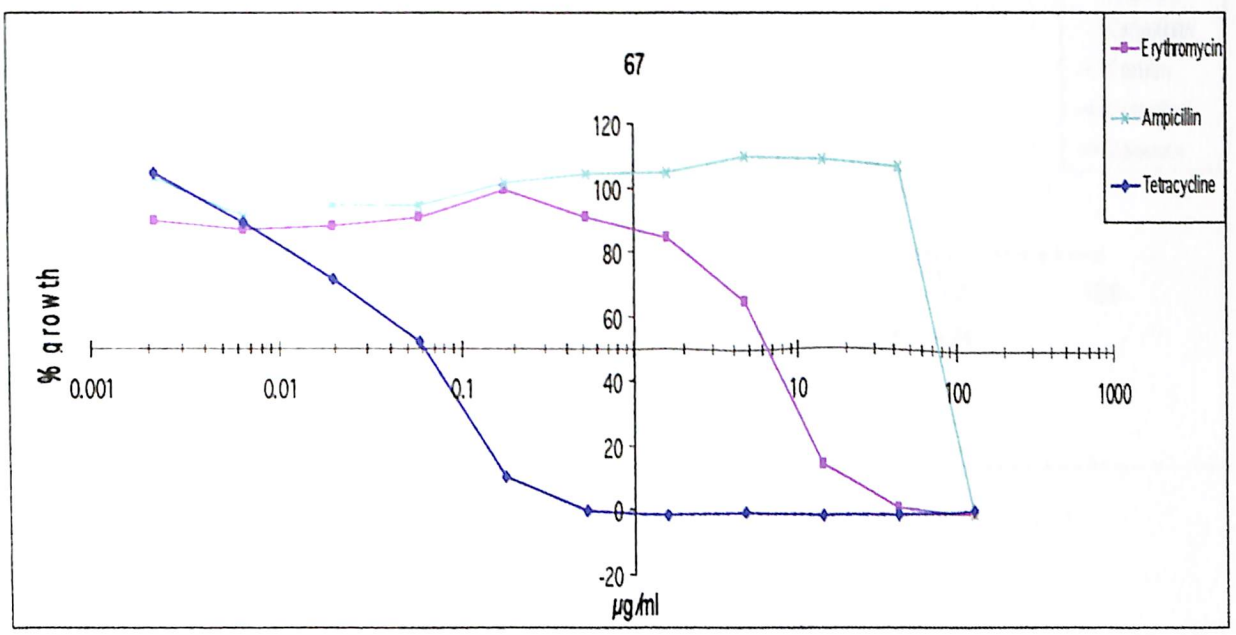
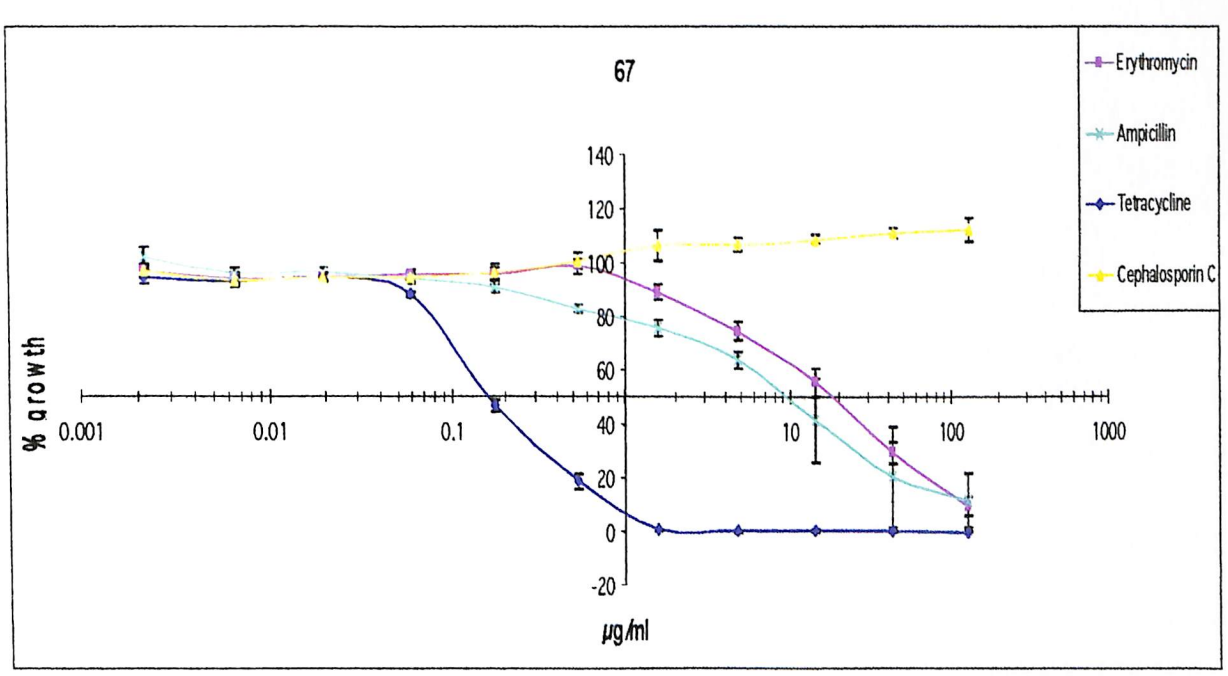


Figure 7.2: Growth curves[a] of 67 showing IC_{50}s for Erythromycin, Ampicillin and Tetracycline at 20.0, 9.5, and 0.15 µg/ml, respectively. Growth curve[a] against Cephalosporin C shows no inhibition by Cephalosporin C.



[a] average of three replicates

Figure 7.3: Growth curves of 67 showing IC_{50}s for Benzylpenicillin, Ampicillin, Carbenicillin, Cefoxitin, Ceftazidime and Aztreonam at 70.0, 55.0, 80.0, 0.015, 0.08, 0.15 and 0.095 µg/ml, respectively.

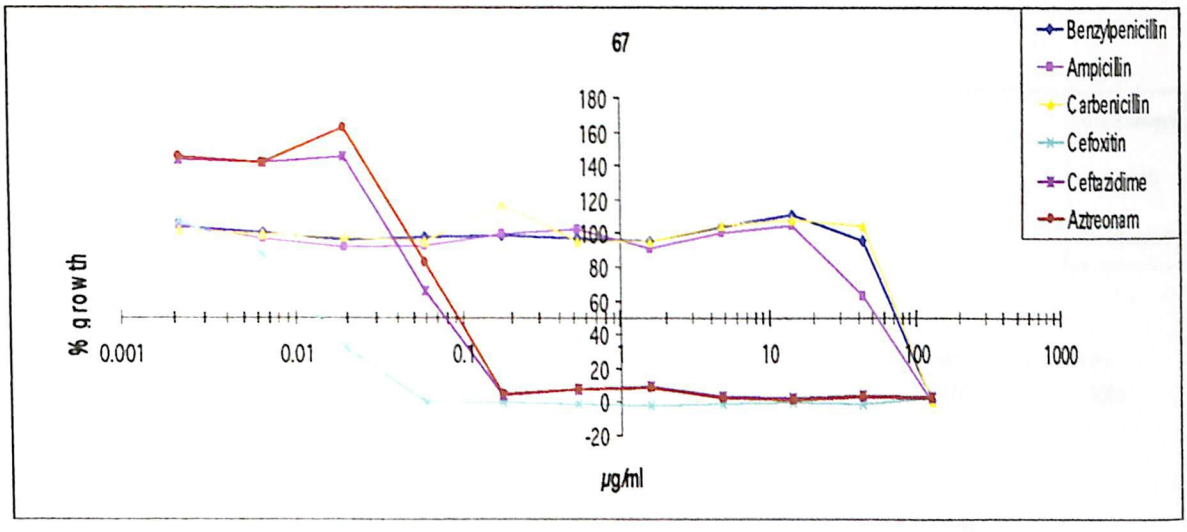


Figure 8. Growth curves of bacterial strain FD1

Figure 8.1: Growth curves of FD1 showing IC_{50}s for Erythromycin, Ampicillin and Tetracycline at 1.0, 100.0, and 0.08 µg/ml, respectively from the general antibiotics susceptibility testing

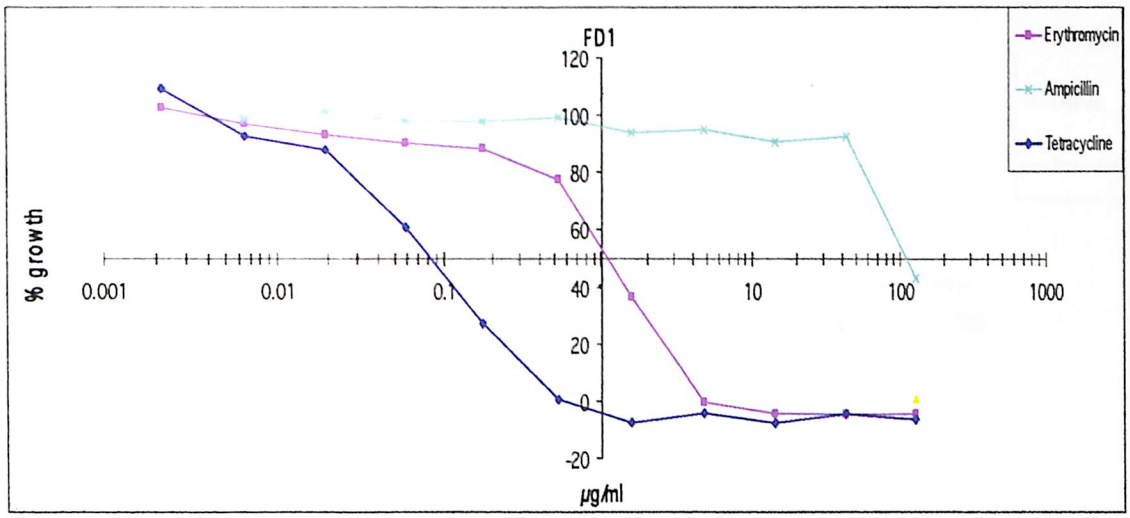
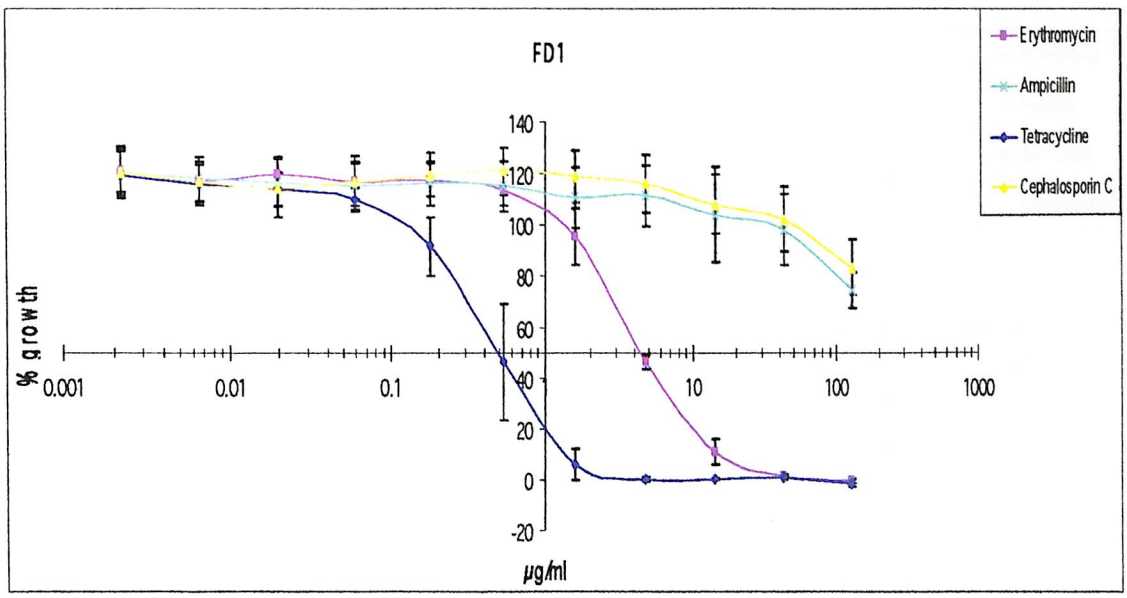


Figure 8.2: Growth curves[a] of FD1 showing IC_{50}s for Erythromycin and Tetracycline at 4.5 and 0.50 µg/ml, respectively. Growth curves[a] against Cephalosporin C and Ampicillin show no inhibition by Cephalosporin C and Ampicillin, respectively.



[a] average of three replicates

Figure 9. Growth curves of bacterial strain 124

Figure 9.1: Growth curves of 124 showing IC_{50}s for Erythromycin, Ampicillin and Tetracycline at 7.0, 75.0, and 0.08 µg/ml, respectively from the general antibiotics susceptibility testing

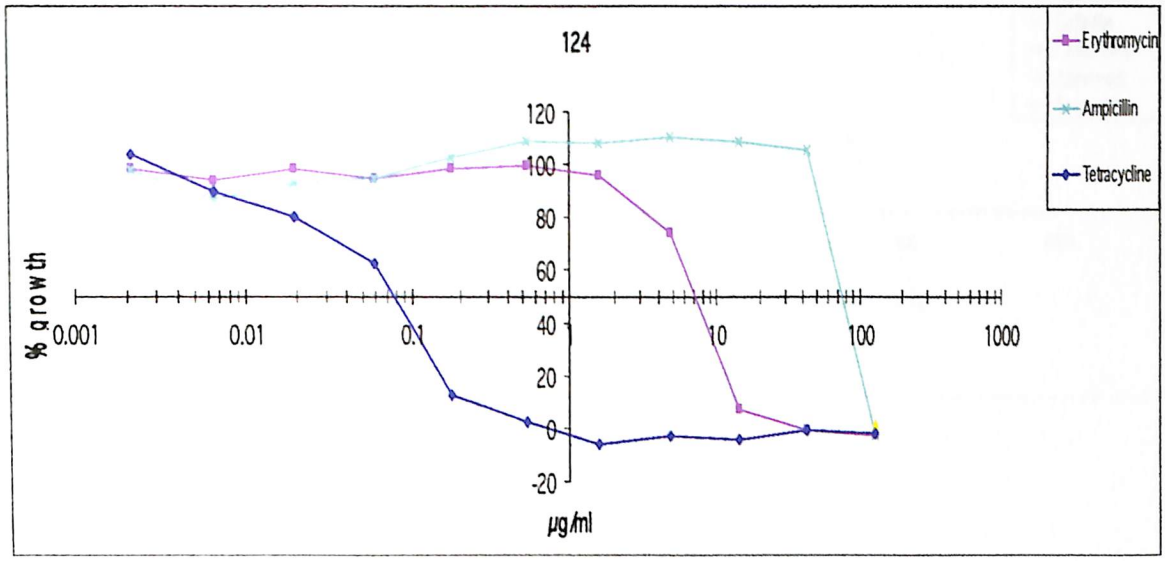
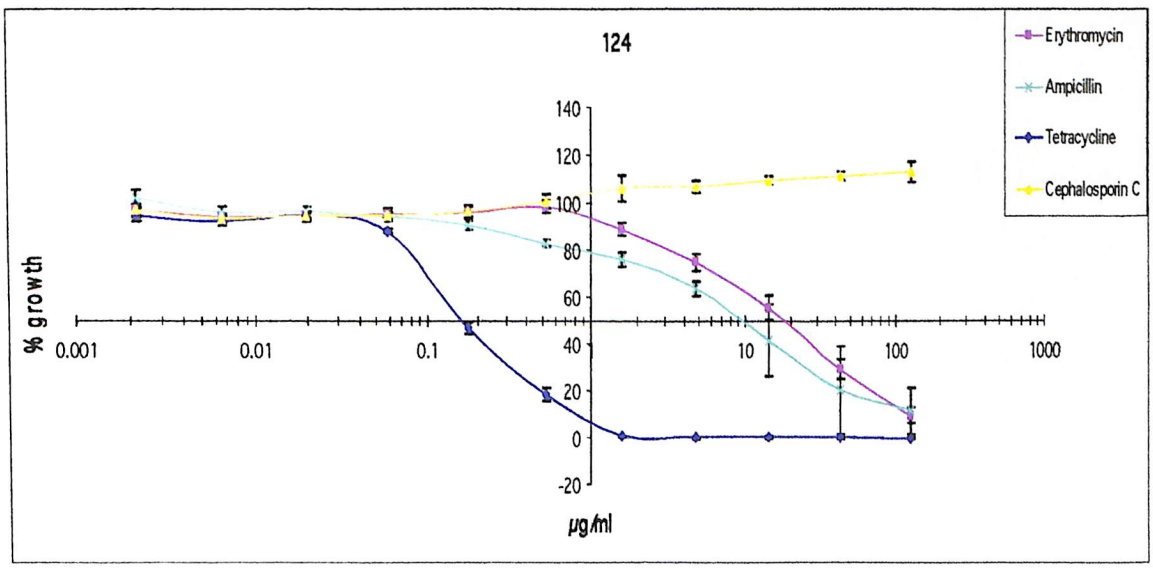
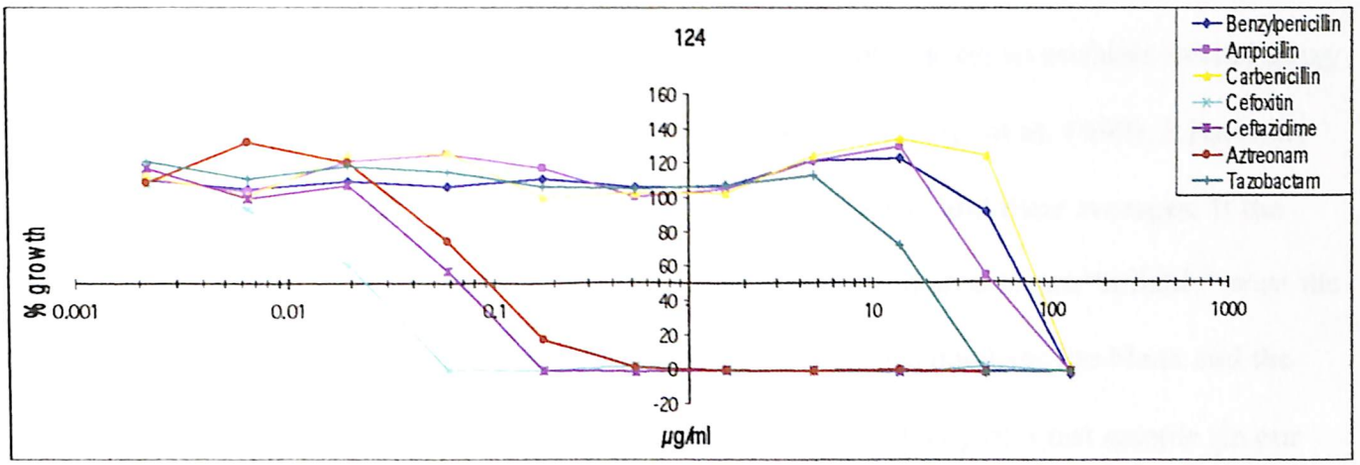


Figure 9.2: Growth curves[a] of 124 showing IC_{50}s for Erythromycin, Ampicillin and Tetracycline at 20.0, 9.5, and 0.15 µg/ml, respectively. Growth curves[a] against Cephalosporin C shows no inhibition by Cephalosporin C.



[a] average of three replicates

Figure 9.3: Growth curves of 124 showing IC_{50}s for Benzylpenicillin, Ampicillin, Carbenicillin, Cefoxitin, Ceftazidime, Aztreonam and Tazobactam at 70.0, 45.0, 80, 0.025, 0.070, 0.09 and 20.0 µg/ml, respectively.



Statistical Analysis:

The data obtained from 0 and 24 hour readings were analyzed using Z prime – factor statistical analysis. Z-prime factor is a unitless parameter to evaluate overall assay quality and validate high throughput screening assays (Zhang, et al. 1999). It basically looks at the deviation around the controls used in the assay and their averages. If the deviations of each control do not overlap with the other, then one can "reliably" trust the data. In other words, if the Z prime-factors of an assay are good for the blank and the negative controls, then when lack of activity or potent activity of a test sample (in our case, the antibiotics) is seen, then the assay is more trustworthy.

The analysis incorporates the average and the standard deviation values for the positive and the negative controls of the assay. The resulting Z prime-factor indicates how reliable the assay is. Screening of the assay quality is categorized by the value of the Z-prime factor of an assay (Zhang, et al., 1999). If the Z prime number is equal to 1, then the assay is an ideal assay. If 1>Z prime > 0.5, then the assay is excellent. For Z prime < 0.5, deviations of the controls touch or overlap (Zhang, et al., 1999). This could be due to contamination in the media, incorrect inocula size or manual faults. A Z'-factor is calculated using the following expression (Zhang, et al., 1999):

$$Z' = 1 - \frac{((3 \times \text{standard deviation of blank}) + (3 \times \text{standard deviation of negative control}))}{|\text{average of blank} - \text{average of negative control}|}$$

Three separate antibiotic susceptibility tests of the 12 environmental isolates were carried out. The preliminary antibiotic susceptibility testing of 12 environmental isolates was done against antibiotics representing common chemical classes (Table 1). Based on the results obtained from this experiment, antibiotic susceptibility of the isolates to

tetracycline, erythromycin and ampicillin were repeated. Cephalosporin C was included in this experiment as well. For the third antibiotic susceptibility testing, the panel of antibiotics used included different generation of β-lactams as well as a β-lactamase inhibitor (Table 1).

RESULTS

Data from first antibiotic susceptibility testing of the environmental isolates showed a high level of resistance towards vancomycin and Linezolid which is expected with gram negative strains. All environmental isolates were extremely susceptible to Ciprofloxacin with complete inhibition of growth (MICs) at ≤0.06μg/ml (Table 3.1). All isolates were susceptible to tetracycline with MICs ≤4.74μg/ml (Table 3.1). Ampicillin susceptibility varied with MICs ranging from 14.22 to 128.0 μg/ml (Table 3.1).

Antibiotic susceptibility testing was repeated for all environmental isolates from the first except for strains FD2, D3, 132 and F5. This time only antibiotics from the classes β-lactams, tetracycline and macrolide were selected (Table 1). The MICs for tetracycline obtained from this testing were consistent with that from the first one (Table 3.1). Tetracycline was effective in all isolates with MICs ≤3.7 μg/ml (Table 3.3). MICs of ampicillin were ≥42.67μg/ml (Table 3.3). All isolates showed significantly high resistance to cephalosporin C with MICs of ≥128.0μg/ml (Table 3.3). 75% of the isolates tested were resistant to erythromycin with MICs > 50μg/ml (Table 3.3).

The third antibiotic susceptibility testing included antibiotics of different generations of β-lactams along with β-lactamase inhibitor and a monobactam (Table 1). The isolates and the control strains were relatively most susceptible to the second and the third generation cephalosporin – cefoxitin and ceftazidime, respectively (Table 3.4 and 3.5). The MICs of cefoxitin were ≤1.58μg/ml (Table 3.4) while that of ceftazidime were ≤14.22μg/ml (Table 3.4). Susceptibility towards benzylpenicillin, ampicillin and carbenicillin was varied with isolates and the control strains. MIC of benzylpenicillin ranged from 0.06-128.0 μg/ml (Table 3.4). MIC of ampicillin ranged from 0.18 to

128.0μg/ml (Table 3.4). MIC of carbenicillin ranged from 0.18μg/ml (Table 3.4). All environmental isolates except strain FD2 were susceptible to aztreonam. Only strains 124, F7 and F18 along with *E. coli* ATCC® 25922 and *E. coli* ATCC® 35218 were tested against tazobactam, the β-lactamase inhibitor. MICs of tazobactam for the control strains were comparable to that of the environmental isolates (Table 3.4 and 3.5).

Table 2. Summary of 50% inhibitory concentration (IC_{50}) of antibiotics used

Table 2.1: Summary of IC_{50} of antibiotics used in the general antibiotic susceptibility testing for the environmental isolates

	Strains											
IC_{50} (μg/ml)of	J2	F18	132	F1	F5	FD2	FD1	D3	67	124	48	F7
Ciprofloxacin	0.004	0.009	0.01	0.003	<	0.0085	0.008	<	<	<	<	<0.049
Gentamycin	0.04	0.050	0.052	0.06	0.035	6.50	6.50	0.003	0.003	0.01	0.065	0.08
Ampicillin	3.50	20.000	10.00	15.00	50.00	70.00	100.00	1.50	1.50	75.00	4.50	-
Chloramphenicol	0.20	0.60	1.00	0.70	0.80	9.00	6.00	0.40	0.40	0.045	0.85	0.25
Tetracycline	0.15	0.40	0.65	0.35	0.7	0.3	0.085	0.05	0.05	0.08	0.30	0.25
Rifampicin	5.50	6.00	-	5.00	9.00	9.00	5.00	0.70	0.70	2.00	7.00	1.50
Erythromycin	15.00	15.00	15.00	15.00	10.00	1.00	1.00	0.90	0.90	7.00	8.00	4.00
Vancomycin	-	-	-	-	-	-	-	-	-	65.00	-	40.00
Neomycin	0.20	0.20	0.20	0.20	0.09	15.00	15.00	0.007	0.007	0.04	0.10	0.15
Kanamycin	0.50	0.50	0.60	0.45	0.35	6.00	6.00	0.02	0.02	0.25	0.50	0.15
Streptomycin	0.90	0.65	0.75	0.70	0.50	65.00	60.00	0.10	0.10	0.25	0.70	0.80
Linezolid	100.00	110.00	-	90.00	-	-	-	100.00	100.00	90.00	-	20.00

< IC_{50} is less than 0.002 μg/ml
- no inhibition

Table 2.2: Summary of IC_{50} of the antibiotics used in general antibiotic susceptibility testing for the reference strains

	Reference Strains		
IC_{50} (µg/ml) of	***E. coli* 25922**	***E. coli* 35218**[a]	***E. cloacae* 13047**
Ciprofloxacin	<0.002	0.003±0[b]	0.004
Gentamycin	0.01	0.06±0.01	0.05
Ampicillin	0.75	-	-
Chloramphenicol	0.45	9.67±0.58	0.80
Tetracycline	0.20	0.28±0.08	0.30
Rifampicin	<	1.67±0.29	4.50
Erythromycin	<	1.27±0.404	9.50
Vancomycin	75.00	-	-
Neomycin	<	0.13±0.043	0.25
Kanamycin	<	0.37±0.057	0.30
Streptomycin	0.01	43.33±10.408	-
Linezolid	50	38.33±20.20	-

[a] average of three replicates with standard deviation
[b] IC_{50} of third replicate <0.002 µg/ml
< IC_{50} is less than 0.002 µg/ml
- no inhibition

Table 2.3: Summary of IC_{50} of antibiotics susceptibility testing for the environmental isolates

Strains	IC_{50}[a] of			
	Erythromycin	Tetracycline	Cephalosporin C	Ampicillin
	μg/ml	μg/ml	μg/ml	μg/ml
F1	18.33±7.6	0.17±0.03	-	27.17±28.8
F18	16.67±5.8	0.28±0.03	-	11.00±3.6
48	18.33±2.9	0.22±0.03	-	7.33±6.7
124	16.67±2.9	0.22±0.03	70.00±17.3	73.33±5.8
F7	10.00±8.9	0.20±0.09	-	6.50[b]
J2	18.33±2.9	0.28±0.08	-	9.00±1.7
67	18.33±2.9	0.25±0	53.33±20.8	75.00±5.0
FD1	4.17±0.3	0.57±0.4	-	-

[a] average of three replicates with standard deviation

[b] data from one replicate only

- no inhibition

Table 2.4: Summary of IC_{50} of β-lactam antibiotics susceptibility testing for the environmental isolates

	IC_{50} of						
Strains	Benzylpenicillin μg/ml	Ampicillin μg/ml	Carbenicllin μg/ml	Cefoxitin μg/ml	Ceftazidime μg/ml	Aztreonam μg/ml	Tazobactam μg/ml
124	70.00	50.00	80.00	0.03	0.07	0.09	20.00
F7	0.004	-	0.8	0.30	4.50	9.50	0.05
F18	8000	8.00	1.50	0.03	0.04	0.02	75.00
FD2	0.03	0.06	0.05	0.20	8.00	85.00	ND
48	-	60.00	8.00	0.08	0.15	0.03	ND
F1	-	6.00	1.50	0.06	0.07	0.02	ND
D3	0.03	3.00	2.00	0.04	0.15	0.04	ND
F5	0.02	0.04	0.20	0.80	10.00	40.00	ND
67	70.00	55.00	80.00	0.02	0.08	0.10	ND

ND No data
- no inhibition

Table 2.5: Summary of IC_{50} of β-lactam antibiotics susceptibility testing for reference strains

	Reference Strains		
IC_{50} (µg/ml)	***E. coli* 25922**	***E.coli* 35218**	***E. cloacae* 13047**
Benzylpenicillin	<	0.003	-
Ampicillin	0.8	-	-
Carbenicllin	0.5	10	85.00
Cefoxitin	0.20	0.20	6.00
Ceftazidime	<	1.50	3.00
Aztreonam	<	1.50	0.08
Tazobactam	0.01	0.06	ND

< IC_{50} is less than 0.002 µg/ml

- no inhibition

ND No data

Table 3. Summary of minimum inhibitory concentration (MIC) of antibiotics

Table 3.1: Summary of MIC of antibiotics used in the general antibiotic susceptibility testing for the environmental isolates

	Strains											
MIC (µg/ml) of	**J2**	**F18**	**132**	**F1**	**F5**	**FD2**	**FD1**	**D3**	**67**	**124**	**48**	**F7**
Ciprofloxacin	0.02	0.06	0.06	0.01	0.002	0.02	0.06	0.01	0.002	0.01	0.002	0.05
Gentamycin	0.18	0.53	0.18	0.53	0.18	14.22	42.67	0.02	0.06	0.18	0.53	0.20
Ampicillin	14.20	42.67	128.00	128.00	128.00	-	-	14.22	128.00	128.00	128.00	50.00
Chloramphenicol	1.58	4.74	14.22	4.74	4.74	128.00	128.00	4.74	0.53	0.53	14.22	0.39
Tetracycline	1.58	4.74	4.74	1.58	4.74	1.58	1.58	0.53	0.53	0.53	1.58	0.39
Rifampicin	-	42.67	-	128.00	42.67	14.22	14.22	4.74	14.22	14.22	42.67	3.13
Erythromycin	-	128.00	128.00	128.00	128.00	4.74	4.74	4.74	42.67	42.70	128.00	6.25
Vancomycin	-	1.58	0.53	0.53	-	-	-	-	-	128.00	-	50.00
Neomycin	1.58	1.58	1.58	4.74	0.18	42.67	42.67	0.02	0.06	0.18	0.53	0.39
Kanamycin	1.58	1.58	4.74	4.74	0.53	14.22	14.22	0.06	0.53	1.58	4.74	0.39
Streptomycin	14.20	-	-	-	0.53	128.00	-	0.53	0.53	0.53	4.74	3.13
Linezolid	-	-	-	-	-	-	-	-	-	-	-	50.00

- no inhibition

Table 3.2: Summary of MIC of antibiotics used in general antibiotic susceptibility testing for the reference strains

MIC (µg/ml) of	Reference Strains		
	E. coli 25922	*E. coli* 35218[a]	*E. cloacae* 13047
Ciprofloxacin	0.01	0.02±0.03	0.007
Gentamycin	0.18	0.29±0.2	0.18
Ampicillin	4.74	-	-
Chloramphenicol	1.58	128.00±0	4.74
Tetracycline	0.53	1.59±0.01	1.58
Rifampicin	0.002	11.05±5.5	14.20
Erythromycin	0.002	20.55±19.8	128.00
Vancomycin	-	-	-
Neomycin	0.18	5.32±7.7	0.53
Kanamycin	0.53	5.79±7.3	1.58
Streptomycin	4.74	128.00[b]	-
Linezolid	-	128.00[b]	-

[a] average of three replicates with standard deviation
[b] two replicates with MIC > 128.0 µg/ml
- no inhibition

Table 3.3: Summary of MIC of antibiotics susceptibility testing for the environmental isolates

	MIC[a] of			
	Erythromycin	Tetracycline	Cephalosporin C	Ampicillin
Strains	μg/ml	μg/ml	μg/ml	μg/ml
F1	128.00[b]	1.58±0	-	71.113±49.3
F7	23.703±16.4	0.527±0	-	-
F18	128[b]	3.687±1.8	-	42.67±0
J2	128[b]	2.634±1.8	-	90.073±65.7
48	128[b]	1.58±0	-	71.113±49.2
67	71.113±49.2	1.23±0.6	128.00±0	128.00±0
124	71.113±49.2	1.23±0.6	128.00±0	128.00±0
FD1	14.22±0	1.23±0.6	-	-

- no inhibition

[a] average of three replicates
[b] MIC of one replicate only; MICs of two replicates >128.0 μg/ml

Table 3.4: Summary of MIC of β-lactam antibiotics susceptibility testing for the environmental isolates

	MIC of						
Strains	Benzylpenicillin μg/ml	Ampicillin μg/ml	Carbenicllin μg/ml	Cefoxitin μg/ml	Ceftazidime μg/ml	Aztreonam μg/ml	Tazobactam μg/ml
124	128.00	128.00	128.00	0.06	0.18	0.53	42.67
F7	-	-	-	0.06	0.18	0.02	42.67
F18	128.00	14.22	4.74	0.06	0.06	0.02	128.00
FD2	0.06	0.18	0.18	0.53	14.22	128.00	ND
48	-	128.00	42.67	0.18	0.18	0.06	ND
F1	-	42.67	4.74	0.18	0.18	0.06	ND
D3	-	14.22	14.22	0.18	0.53	0.06	ND
F5	0.18	0.53	0.18	1.58	14.22	0.53	ND
67	128.00	128.00	128.00	0.06	0.18	0.18	ND

ND No data
- no inhibition

Table 3.5: Summary of MIC of β-lactam antibiotics susceptibility testing for reference strains

	Reference Strains		
MIC of (µg/ml)	***E. coli* 25952**	***E. coli* 35218**	***E.cloacae* 13047**
Benzylpenicllin	42.67	-	-
Ampicillin	14.22	-	-
Carbenicillin	14.22	-	-
Cefoxitin	0.18	0.06	14.22
Ceftazidime	0.18	0.18	14.22
Aztreonam	0.18	0.06	1.58
Tazobactam	128.00	42.67	ND

- no inhibition

ND No data

Table 4. Summary of minimum bactericidal concentration (MBC) of antibiotics used

Table 4.1: Summary of MBCof antibiotics used in the general antibiotic susceptibility testing for the environmental isolates

	Strains											
MBC (µg/ml) of	**J2**	**F18**	**132**	**F1**	**F5**	**FD2**	**FD1**	**D3**	**67**	**124**	**48**	**F7**
Ciprofloxacin	0.06	0.05	14.22	0.17	0.020	0.06	0.06	0.01	0.002	0.53	0.002	0.05
Gentamycin	0.53	0.53	0.53	14.20	0.18	42.67	-	0.20	0.06	4.74	0.53	0.78
Ampicillin	128.00	42.70	128.00	128.00	128.00	-	-	128.00	128.00	128.00	-	-
Chloramphenicol	-	-	-	-	-	-	-	42.67	-	-	-	3.13
Tetracycline	128.00	-	-	128.00	128.00	4.74	1.58	1.58	-	-	-	6.25
Rifampicin	-	-	-	-	-	-	-	14.22	-	-	-	3.13
Erythromycin	-	-	-	-	-	14.22	42.67	14.22	-	-	-	50.00
Vancomycin	-	-	-	-	-	-	-	-	-	-	-	-
Neomycin	1.58	14.20	0.53	0.53	0.18	128.00	128.00	0.02	14.22	0.53	0.53	6.25
Kanamycin	1.58	4.74	1.58	4.74	1.58	42.67	42.67	0.18	4.74	14.22	14.22	3.13
Streptomycin	14.22	4.74	14.20	4.74	4.74	-	-	0.527	1.5803	0.5267	4.7407	12.5
Linezolid	-	-	-	-	-	-	-	-	-	-	-	-

- no inhibition

Table 4.2: Summary of MBC of antibiotics used in general antibiotic susceptibility testing for the reference strains

	Reference Strains		
MBC (µg/ml) of	***E. coli* 25922**	***E. coli* 35218**[a]	***E. cloacae* 13047**
Ciprofloxacin	0.01	0.06±0.093	0.020
Gentamycin	0.18	0.88±0.607	0.53
Ampicillin	4.74	-	-
Chloramphenicol	128.00	-	-
Tetracycline	128.00	128.00[b]	-
Rifampicin	0.010	-	-
Erythromycin	-	-	-
Vancomycin	-	-	-
Neomycin	0.18	2.17±2.37	0.53
Kanamycin	0.53	2.63±1.82	1.58
Streptomycin	4.7407	-	-
Linezolid	-	-	-

[a] average of three replicates with standard deviation
[b] MBC of one replicate only; MBC of two replicates >128.0 µg/ml
- no inhibition

Table 4.3: Summary of MBC of antibiotics susceptibility testing for the environmental isolates

	MBC[a] of			
Strains	Erythromycin µg/ml	Tetracycline µg/ml	Cephalosporin C µg/ml	Ampicillin µg/ml
F1	-	128.0±0	-	128.0±0
F7	33.187±16.4	1.932±2.4	-	-
F18	-	128.0±0	-	42.67±0
J2	-	128.0±0	-	128.0±0[b]
48	-	99.56±49.3	-	128.0[c]
67	-	128.0±0[b]	128.00±0	128.0±0
124	-	128.0[c]	128.00[b]	128.0±0
FD1	42.67[c]	71.113±49.2	-	-

- no inhibition

[a] average of three replicates

[b] MBC of two replicates only; 3rd MBC value >128.0µg/ml

[c] MBC of one replicate only; 1st & 2nd MBC values >128.0 µg/ml

Table 4.4: Summary of MBC of β-lactam antibiotics susceptibility testing for environmental isolates

	MBC of						
Strains	Benzylpenicillin µg/ml	Ampicillin µg/ml	Carbenicllin µg/ml	Cefoxitin µg/ml	Ceftazidime µg/ml	Aztreonam µg/ml	Tazobactam µg/ml
124	128.00	128.00	-	1.58	0.18	0.53	42.67
F7	-	-	-	0.06	0.18	0.18	128.00
F18	-	42.67	4.74	0.18	0.18	0.02	-
FD2	0.06	0.18	0.18	0.53	42.67	128.00	ND
48	-	-	-	42.67	4.74	0.06	ND
F1	-	42.67	128.00	4.74	4.74	4.74	ND
D3	-	128.00	14.22	0.18	0.53	0.06	ND
F5	0.53	0.53	1.58	4.74	128.00	-	ND
67	-	-	-	4.74	0.53	14.22	ND

ND No data
- no inhibition

Table 4.5: Summary of MBC of β-lactam antibiotics susceptibility testing for reference strains

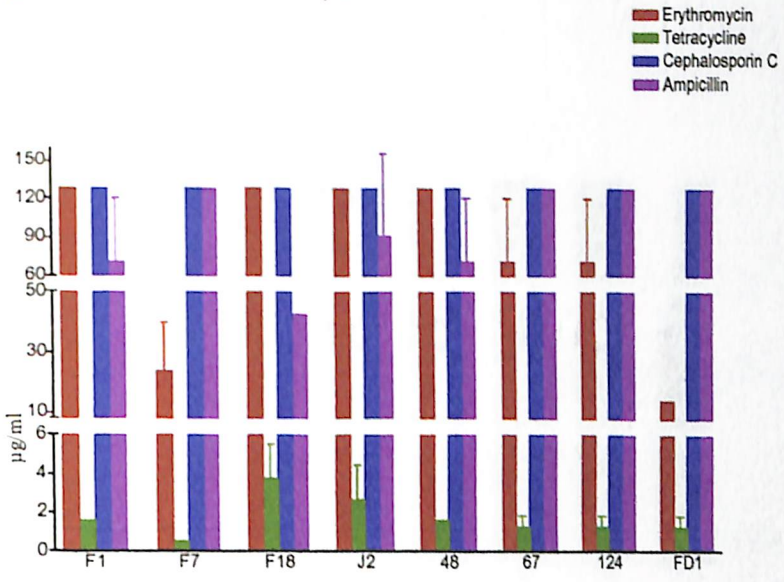
	Reference Strains		
MBC of (µg/ml)	***E. coli* 25922**	***E. coli* 35218**	***E.cloacae* 13047**
Benzylpenicllin	42.67	-	-
Ampicillin	14.22	-	-
Carbenicillin	14.22	-	-
Cefoxitin	1.58	0.06	14.22
Ceftazidime	0.18	0.18	14.22
Aztreonam	0.06	0.18	1.58
Tazobactam	128.00	42.67	ND

- no inhibition

ND No data

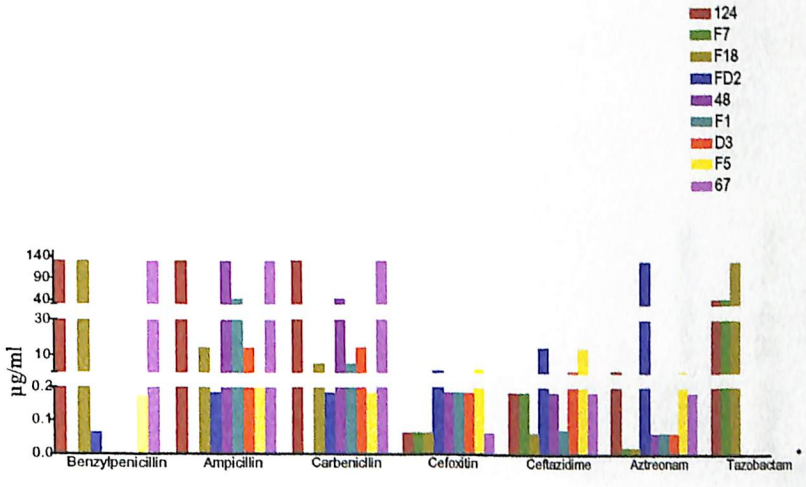
Figure 10. Minimum Inhibitory Concentration

Figure 10.1: Minimum Inhibitory Concentration* of four antibiotics



* average of three replicates

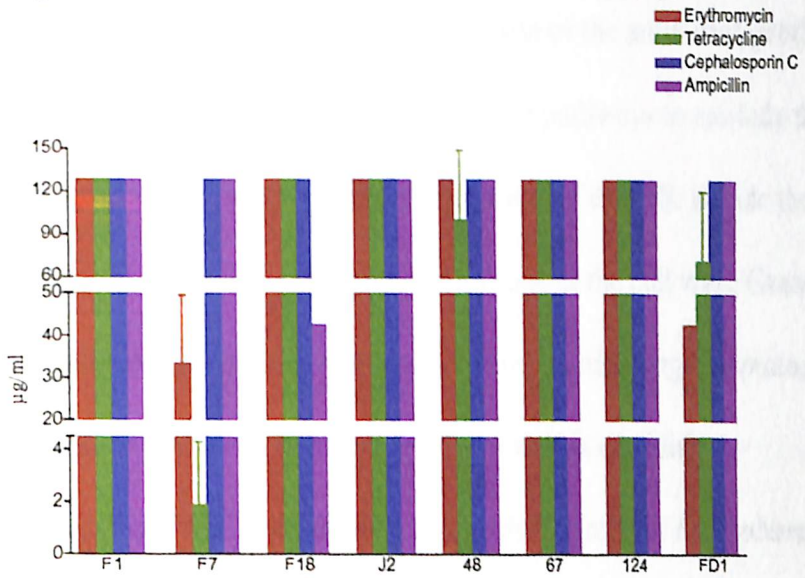
Figure 10.2: MIC of β-lactam antibiotics susceptibility testing



*MIC data not available for F18, FD2, 48, F1, D3, F5 and 67

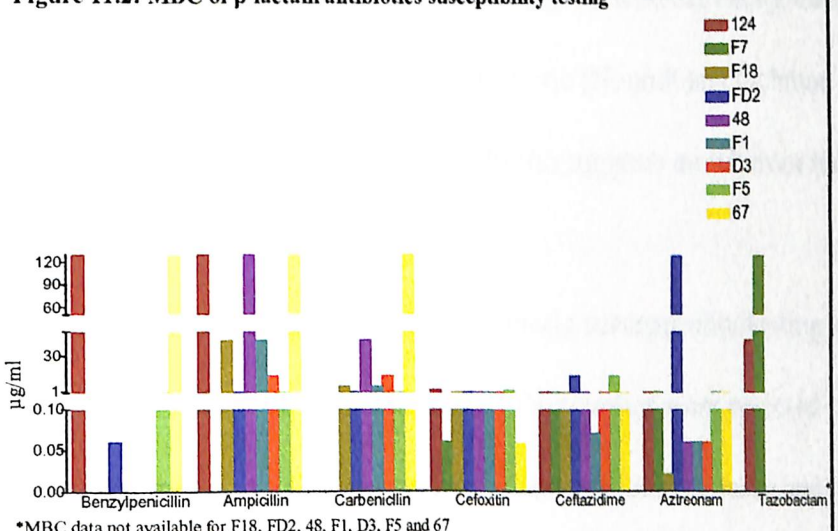
Figure 11. Minimum Bactericidal Concentraion

Figure 11.1: Minimum Bactericidal Concentration* of four antibiotics



* average of three replicates

Figure 11.2: MBC of β-lactam antibiotics susceptibility testing



*MBC data not available for F18, FD2, 48, F1, D3, F5 and 67

DISCUSSION

Antibiotic resistance by bacteria can be an intrinsic or an acquired property. In either case, it enables bacteria to modify the target site of the antibiotic, produce an enzyme that modifies the antibiotic, alter biochemical pathways to exclude the antibiotic and develop mechanisms to transport the antibiotic out of the cell. Beside these mechanisms, bacteria's chief defense against antibiotic is the cell wall. Gram-negative bacteria are especially resistant to β-lactam antibiotics, which target peptidoglycan synthesis in cell wall of bacteria, because their cell-wall is diminished.

NCCLS specified MIC interpretive standards (μg/ml) for *Enterobacteriaceae* was used in this antimicrobial testing to determine the level of antibiotic susceptibility of the isolates (NCCLS M100-S12 2002). In addition, results of this study were also compared with Houndt and Ochmans' determination of high-level resistance to antibiotics and background levels of antibiotic resistance. According to Houndt, in general, high-level resistance means resistance to concentrations of >50 μg/ml, whereas background levels were less than 10 to 20μg/ml depending on the antibiotic (Houndt and Ochman 2000). Background level of resistance to a particular antibiotic suggests an inherent nature of bacteria to resist that antibiotic.

The environmental isolates selected for antibiotic susceptibility testing are classified as being gram-negatives. A broad range of antibiotics were selected for antibiotic susceptibility testing of 12 environmental isolates (see Methods and Materials) to determine the pattern of antibiotic resistance of these isolates. According to NCCLS definition and specification, 83.3% of the isolates tested were found to be resistant towards ampicillin. 80.0% of the isolates found to be resistant to ampicillin showed a

high level of resistance to ampicillin. The remaining 12.3% of the isolates still showed background level of resistance to ampicillin. 92% of the isolates were found to show high level of resistance to linezolid which is expected of gram negative bacteria. After ampicillin, high level of antibiotic resistance was seen in decreasing order towards vancomycin, erythromycin, streptomycin, rifampicin and chlormaphenicol. The result of this antibiotic susceptibility testing suggests that perhaps there is a general presence of multidrug resistance pattern. On the contrary, 75% of the isolates were susceptible to tetracycline. The remaining 25% of the isolates were designated as being intermediate in terms of resistance to tetracycline. Many earlier studies have shown that bacteria resistant to tetracycline are capable of tetracycline efflux (Levy 1984) which enables the bacteria to resist the entry of the drug into the bacterial cell. Therefore, it could be that the environmental isolates not susceptible to tetracycline are capable of tetracycline efflux. Although there are reports suggesting alteration of tetracycline target site (ribosome), the exclusive mechanism for tetracycline resistance has involved decreased penetration of the drug into the cell (Levy 1984).

With this result at hand, a second round of antibiotic susceptibility testing was performed. This time 8 out of the 12 prior isolates were tested against tetracycline, ampicillin, erythromycin and cephalosporin C. These antibiotics represent the common classes of antibiotics. Resistance to ampicillin was observed as anticipated from previous results. Growth inhibition of 75% environmental isolates due to erythromycin was seen only in higher concentrations of erythromycin (Table 3.3). This portion of the isolates showed high-level of resistance to erythromycin. All environmental isolates tested were susceptible to tetracycline (Table 3.3). In addition, growth inhibition of all environmental

isolates due to cephalosporin C was seen at the highest test concentration (128.0μg/ml) or greater (Table 3.3). Cephalosporin C is the parent compound of a number of semi-synthetic antibiotics that are used in the treatment of infections due to gram-positive and gram-negative bacteria. Again, the high level of resistance to cephalosporin C, ampicillin and erythromycin could suggest the presence of a multidrug resistance pattern in these environmental isolates.

To evaluate if the environmental isolates showed any difference in resistance level to different generation of β-lactams, we performed another antibiotic susceptibility testing. Accordingly, this test included different generation of β-lactams as well as a monobactam (Aztreonam) and a β-lactamase inhibitor (Tazobactam).

Resistance to ampicillin, benzylpenicillin and carbenicillin was observed as expected of previous results. 77.8%, 44.4% and 33.3% of the environmental isolates tested were found to exhibit high-level resistance to benzylpenicillin, ampicillin and carbenicillin, respectively. This result suggests the possibility of a multidrug resistance pattern among the environmental isolates. 100% of the isolates were found to be susceptible to cefoxitin. Cefoxitin possess a significant gram-negative and gram-positive activity although a significant number of strains including *Enterobacter cloacae* are now resistant (Fisher, 1984). Cefoxitin falls in the second generation of cephalosporin. Similarly, 77.77% of the environmental isolates tested were susceptible to ceftazidime, with the remaining 23.23% still below the resistance level. Ceftazidime is a third generation cephalosporin. Ceftazidime is a potent broad-spectrum antibiotics exhibiting high stability to β-lactamases, may also be a β-lactamase inhibitor (Bush and Sykes 1984). The primary basis for the expanded spectrum is improved β-lactamase stability

(Birnbaum et al. 1978). Susceptibility to aztreonam was also observed. Aztreonam is a potent antimicrobial agent as well as β-lactamase inhibitors (Bush and Sykes 1984). Tazobactam displayed less inhibitory effect on the environmental isolates. Tazobactam is a β-lactamase inhibitor. However in this study it was not used synergistically with a β-lactam. Therefore, there was no β-lactam substrate for β-lactamase, if any produced by the isolates, to act on. Tazobactam does not have any hydrolyzing capability. Hence, majority of the isolates showed growth in the presence of tazobactam.

The consistent trend of resistance to ampicillin and earlier generation of β-lactams (such as Cephalosporin C) suggests that the environmental isolates tested are resistant against β-lactams, at least the earlier generations of β-lactams. β-lactam is an important class of antibiotics because it is widely used for clinical treatment of infections. Resistance to β-lactams has been attributed to the production of β-lactamase enzymes by bacteria and in recent years to the extended-spectrum-β-lactamase enzymes. The β-lactamase enzyme hydrolyzes the β-lactam ring and renders the effect of these antibiotics.

There are different generations of β-lactams available based on their antimicrobial activity and resistance to β-lactamase. Bulkier β-lactam ring is a characteristic of subsequent generations of β-lactams. The additional steric hindrance around the β-lactam ring provides a protective shield against specificity of the hydrolytic action of β-lactamase. The environmental isolates tested were resistant to simpler β-lactams but appeared relatively susceptible to the newer generation of β-lactams. The level of resistance suggests acquired resistance. Further testing needs to be done to asses if the gene encoding β-lactamase enzyme is intrinsic or acquired.

In this study, the environmental pool of resistance was assessed among rhizosphere bacteria because genetic exchange and transfer of antibiotic resistant genes may be enhanced in the rhizosphere (Halda-Alija, et al. 2000). Establishing an antibiotic resistance pattern among enteric bacteria in the environment could be potentially used as warning of possible contamination and as bio-index of water and soil/sediment quality deterioration. This will significantly contribute to the long-term protection of human health and freshwater wetlands.

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