

## Antibiotic Sensitivity Test for Environmental Bacteria (*Brevibacillus brevis*, *Bacillus coagulans*, *Enterococcus raffinosus*, and *Macrocooccus caseolyticus*)

Dareen El Shareef Ahmed<sup>1\*</sup>, Hussein Yassine<sup>2</sup>, Hana Gadalla<sup>3</sup> and Ana Janic<sup>4</sup>

<sup>1</sup>M.S. in Microbiology, Faculty of Pharmacy, Benghazi University, Benghazi, Libya

<sup>2</sup>Dental Student, School of Dentistry, University of Detroit Mercy, Detroit, Michigan, USA

<sup>3</sup>Private Practice Periodontist Metro Detroit Area, USA

<sup>4</sup>Assistant Professor, Division of Clinical Dentistry, School of Dentistry, University of Detroit Mercy, Detroit, Michigan, USA

\*Corresponding Author: Dareen El Shareef Ahmed, M.S. in Microbiology, Faculty of Pharmacy, Benghazi University, Benghazi, Libya.

Received: February 12, 2020; Published: May 21, 2020

### Abstract

Four gram positive bacteria were isolated from the aerosol of Al Marj City from December 2012 through March 2013, because there was no research related to this subject previously completed. The four bacteria evaluated were as follows: *Brevibacillus brevis*, *Bacillus coagulans*, *Enterococcus raffinosus* and *Macrocooccus caseolyticus*. They were screened for sensitivity to twelve antibiotics by the Bauer disc diffusion method. The highest sensitivity was recorded to Chloramphenicol, Ciprofloxacin, and Amikacin. High resistance was observed for *Macrocooccus caseolyticus* in Colistin sulphate, Ampicillin, Oxacillin, Cephalexin, Cefoxitin and Augmentin. Highest sensitivity recorded for *Macrocooccus caseolyticus* with Chloramphenicol, Ciprofloxacin, Erythromycin, Gentamycin, Tobramycin and Amikacin. High resistance was observed in Colistin sulphate, Erythromycin, Oxacillin, Cephalexin and Cefoxitin and higher sensitivity performed for *Brevibacillus brevis* by Chloramphenicol, Ciprofloxacin, Augmentin and Amikacin. High resistance was observed in Oxacillin, Cephalexin and Cefoxitin and higher sensitivity performed for *Bacillus coagulans* by Chloramphenicol, Ciprofloxacin, Augmentin, Erythromycin and Amikacin. Of these, the highest sensitivity was recorded with Augmentin. High resistance was recorded for Colistin sulphate. By means of antibiotic sensitivity test, highest resistance was recorded to *Brevibacillus brevis* and the least resistance was recorded to *Macrocooccus caseolyticus*. The combination of antibiotic Amoxicillin/Clavulanic acid (Augmentin) was found to be the most effective antibiotic with highest sensitivity recorded for three environmental bacteria, which are *Brevibacillus brevis*, *Bacillus coagulans* and *Enterococcus raffinosus*.

**Keywords:** *Brevibacillus brevis*; *Bacillus coagulans*; *Enterococcus raffinosus*; *Macrocooccus caseolyticus*

### Introduction

An antibiotic, meaning “against life”, is defined as a lower molecular weight chemical substance produced by one microorganism that is destructive to, and active against another microorganism [1]. The word antibiotic came from the word antibiosis, a term coined in 1889 by Louis Pasteur’s pupil, Paul Vuillemin, meaning a process by which life could be used to destroy life. Antibiotics are one of the greatest discoveries in the history of medicine and have a profound effect on the human life.

### Classification of antibiotics

There are several classification schemes for antibiotics, based on bacterial spectrum (broad versus narrow), route of administration (injectable versus oral versus topical), or type of activity (bactericidal versus bacteriostatic). The most useful is based on chemical structure. Antibiotics within a structural class will generally show similar patterns of effectiveness, toxicity and allergic potential. The

antimicrobial drugs grouped by structure and function to the five modes of action cover most antibiotics. Figure 1 describes the modes of action of antibiotics.

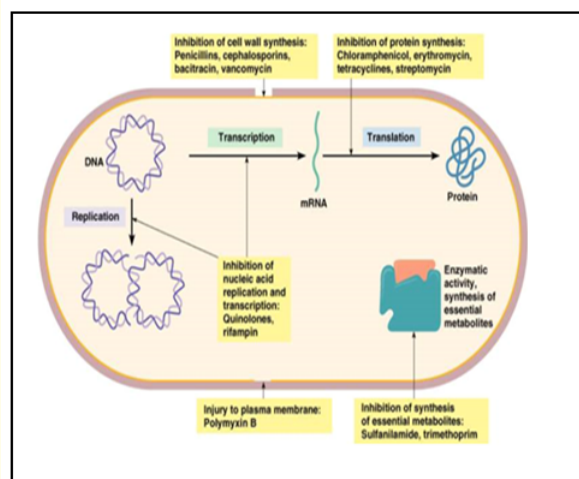


Figure 1: Mechanisms of action of antibiotics. <http://dc130.4shared.com>.

### Cell wall synthesis inhibitor

$\beta$ -Lactam antibiotic (bactericidal) are a broad class of antibiotics, consisting of all antibiotic agents that contains a  $\beta$ -lactam ring in their molecular structures. This includes penicillin derivatives (penams), cephalosporins (cephems), monobactams and carbapenems [2]. Most  $\beta$ -lactam antibiotics work by inhibiting cell wall biosynthesis in the bacterial organism and are the most widely used group of antibiotics. Up until 2003, when measured by sales, more than half of all commercially available antibiotics in use were  $\beta$ -lactam compounds [3].

$\beta$ -Lactam antibiotics are bactericidal and act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity, especially in Gram positive organisms, being the outermost and primary component of the wall. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by DD-transpeptidases, which are penicillin-binding proteins (PBPs). PBPs vary in their affinity for binding penicillin or other  $\beta$ -lactam antibiotics. The amount of PBPs varies among bacterial species [4]. The  $\beta$ -Lactam antibiotics used in this study were ampicillin, oxacillin, amoxicillin/clavulanic acid, cephalixin and cefoxitin, as shown in figure 2.

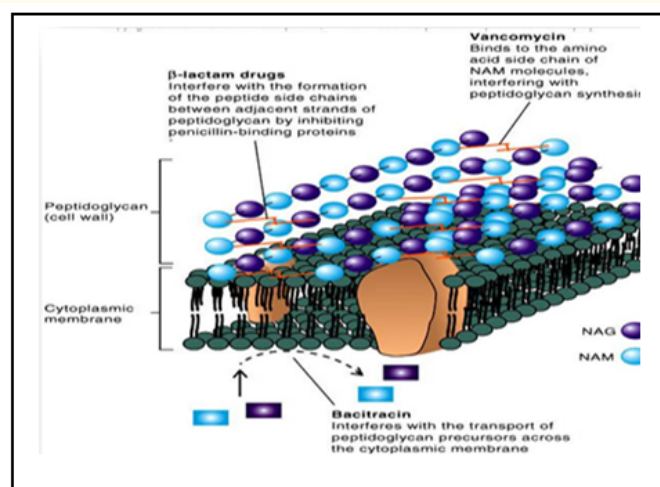


Figure 2: The cell wall inhibitor antibiotics. <http://www.pc.maricopa.edu>.

## Inhibitors of protein synthesis

### Macrolides

Macrolides are a group of protein synthesis inhibitor antibiotics, whose activity stems from the presence of a large macrocyclic lactone ring. The mechanism of action of macrolides is inhibition of bacterial protein biosynthesis, and they are thought to do this by preventing peptidyl transferase from adding the growing peptide attached to tRNA to the next amino acid, as well as inhibiting ribosomal translocation [6]. Another potential mechanism is premature dissociation of the peptidyl-tRNA from the ribosome [7]. The macrolide protein synthesis antibiotic used in this study was erythromycin antibiotic, as shown in figure 3.

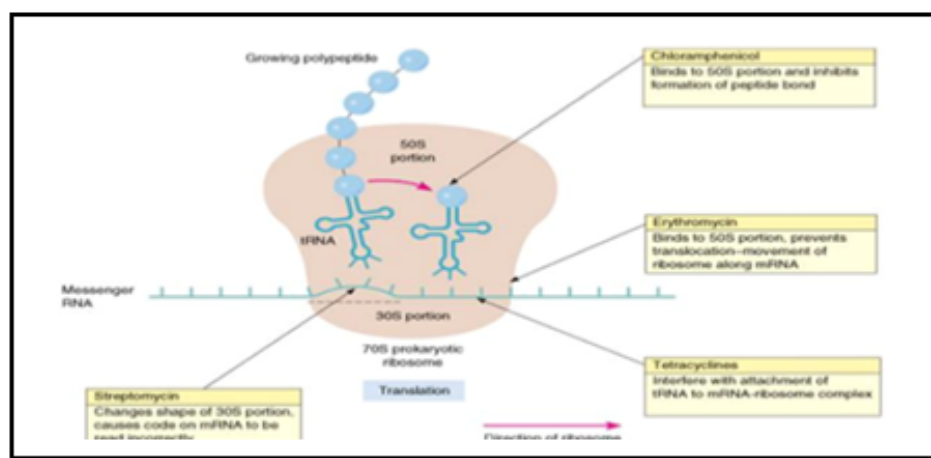


Figure 3: Inhibitors of protein synthesis antibiotics. <http://www.pc.maricopa.edu>.

### Phenocols

Chloramphenicol is a bacteriostatic antimicrobial that became available in 1949. It is considered a prototypical broad-spectrum antibiotic that is effective against a wide variety of Gram positive and Gram negative bacteria [8]. Chloramphenicol was originally derived from the bacterium *Streptomyces venezuelae*, isolated by David Gottlieb and introduced into clinical practice in 1949. Chloramphenicol is a bacteriostatic drug that inhibits bacterial growth by blocking protein synthesis. Chloramphenicol prevents protein chain elongation by inhibiting the peptidyl transferase activity of the bacterial ribosome. It specifically binds to A2451 and A2452 residues in the 23S rRNA of the 50S ribosomal subunit, preventing peptide bond formation. While chloramphenicol and the macrolide class of antibiotics both interact with ribosomes, chloramphenicol is not a macrolide. It directly interferes with substrate binding, whereas macrolides sterically block the progression of the growing peptide [9].

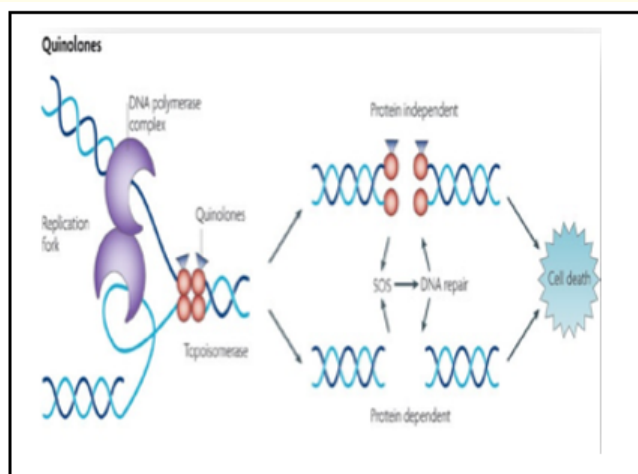
### Aminoglycoside

An aminoglycoside is a molecule or a portion of a molecule composed of amino-modified sugars [10]. Several aminoglycosides, such as amikacin, arbekacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, rhodostreptomycin [11], streptomycin, tobramycin and apramycin, function as antibiotics that are effective against various types of bacteria. Aminoglycosides that are derived from bacteria of the *Streptomyces* genus are named with the suffix “mycin”, whereas those that are derived from *Micromonospora* are named with the suffix

“micin” [12]. Aminoglycosides have several potential antibiotic mechanisms, including inhibitors of protein synthesis, although their exact mechanism of action is not fully known. They may interfere with the proofreading process of protein synthesis, causing increased rate of error with premature termination. Evidence demonstrates the inhibition of ribosomal translocation where the peptidyl-tRNA moves from the A-site to the P-site [7]. They bind to the bacterial 30S ribosomal subunit [13]. Since they can also disrupt the integrity of bacterial cell membrane, they are known as both bacteriostatic and bactericidal agents.

### **Inhibitors of DNA replications**

The quinolones are a family of synthetic broad-spectrum antibacterial drugs [14] that exert their bactericidal effect by interfering with bacterial DNA synthesis and replication. Many quinolones antibiotics belong to a subgroup called fluoroquinolones, which have a fluoro functional group associated with the molecule. Both terms are therefore used to describe antibiotics in this class. Fluoroquinolones inhibit the topoisomerase II ligase domain, leaving the two nuclease domains intact. This modification, coupled with the constant action of the topoisomerase II in the bacterial cell, leads to DNA fragmentation via the nuclease activity of the intact enzyme domains. Recent evidence has shown eukaryotic topoisomerase II is also a target for a variety of quinolone-based drugs. Thus far, most of the compounds that show high activity against the eukaryotic type II enzyme contain aromatic substituents at their C-7 positions [15]. Figure 4 describes inhibitors of nucleic acid synthesis antibiotics.



**Figure 4:** The nucleic acid synthesis inhibitor antibiotics. <http://www.nature.com/nrmicro/journal/v8/n6/full/nrmicro2333.html>.

### **Inhibitors of bacterial cell membrane function**

Polymyxins (Lipopeptides) are antibiotics with a general structure consisting of a cyclic peptide with a long hydrophobic tail [16]. They disrupt the structure of the bacterial cell membrane by interacting with its phospholipids. They are produced by non-ribosomal peptide synthetase systems in Gram positive bacteria such as *Paenibacillus polymyxa* [17]. They are also selectively toxic for Gram negative bacteria due to their specificity for the lipopolysaccharide molecule that exists within many Gram negative outer membranes. Figure 5 describes the cell membrane function inhibitor antibiotics.

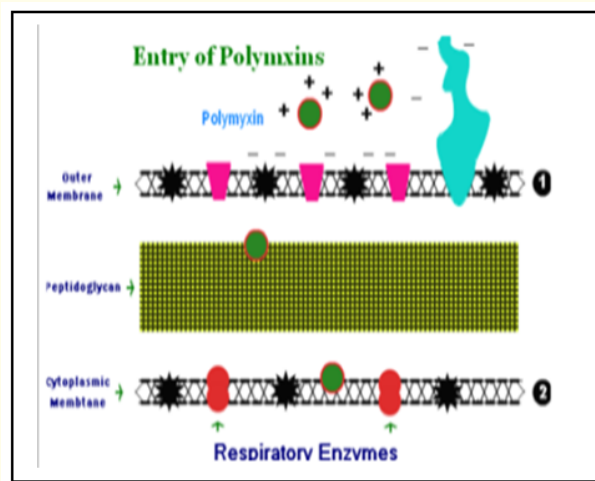


Figure 5: The cell membrane function inhibitor antibiotics. <http://chemistry.tutorvista.com/biochemistry/antibiotics.html>.

### The environmental bacteria

Environmental bacteria are the bacteria live in environments like fresh water, oceans, lakes, rivers, soil, and in the air. In this research we studied the following environmental bacteria: *Brevibacillus brevis*, *Bacillus coagulans*, *Enterococcus raffinosus*, and *Macrocooccus caseolyticus*.

### *Bacillus coagulans*

*Bacillus coagulans* is a lactic acid-forming bacterial species within the genus *Bacillus*. The organism was first isolated and described as *Bacillus coagulans* in 1915 by B.W. Hammer at the Iowa Agricultural Experiment Station as a cause of an outbreak of coagulation in evaporated milk packed by an Iowa condensary [18]. Separately isolated in 1935 and described as *Lactobacillus sporogenes* in the Fifth edition of Bergey's Manual, it exhibits characteristics typical of both *Lactobacillus* and *Bacillus*. Its taxonomic position between the families Lactobacillaceae and Bacillaceae was often debated.

*Bacillus coagulans* is a Gram positive rod that is 0.9  $\mu\text{m}$  by 3.0  $\mu\text{m}$  to 5.0  $\mu\text{m}$  in size. It is a catalase positive, spore-forming, motile, facultative anaerobe. *Bacillus coagulans* may appear Gram negative when entering the stationary phase of growth. The optimum temperature for its growth is 50°C (122°F) but can tolerate temperatures between 30°C - 55°C (86 - 131°F). IMViC tests VP and MR (methyl-red) tests are positive. One strain of this bacterium has also been assessed for safety as a food ingredient [19]. Spores are activated in the acidic environment of the stomach and begin germinating and proliferating in the intestine. Spore forming *Bacillus coagulans* are used in some countries as probiotic for patients on antibiotics.

### *Brevibacillus brevis*

*Bacillus brevis* also known as *Brevibacillus brevis* is a Gram positive aerobic spore forming bacillus commonly found in soil, air, water and decaying matter. It is rarely associated with infectious diseases. *Bacillus brevis* has optimum growing temperatures of 35°C - 55°C. It is a motile spore forming bacteria with positive catalase activity, amylase negative, casein negative, gelatinase positive, indole negative, VP negative and most are citrate utilizers. *Bacillus brevis* was reclassified to *Brevibacillus brevis* [20].

### *Enterococcus raffinosus*

*Enterococcus* is a genus of lactic acid bacteria of the phylum Firmicutes. *Enterococci* are Gram positive cocci that often occur in pairs (diplococci) or short chains and are difficult to distinguish from streptococci using physical characteristics alone. *Enterococci* are facultative anaerobic organisms, meaning, they are capable of cellular respiration in both oxygen-rich and oxygen-poor environments [21]. Though they are not capable of forming spores, *Enterococci* are tolerant to a wide range of environmental conditions, including extreme temperatures (10 - 45°C), pH (4.5 - 10.0) and high sodium chloride concentrations. *Enterococci* typically exhibit gamma-hemolysis on sheep's blood agar [22]. Since drinking water contaminated by *Enterococci* can be harmful and capable of causing nosocomial and community acquired infections, a work was conducted with the objective of studying the species distribution of *Enterococcus* in various water sources, as well as their resistance to antibiotics and disinfectants.

### *Macrocooccus caseolyticus*

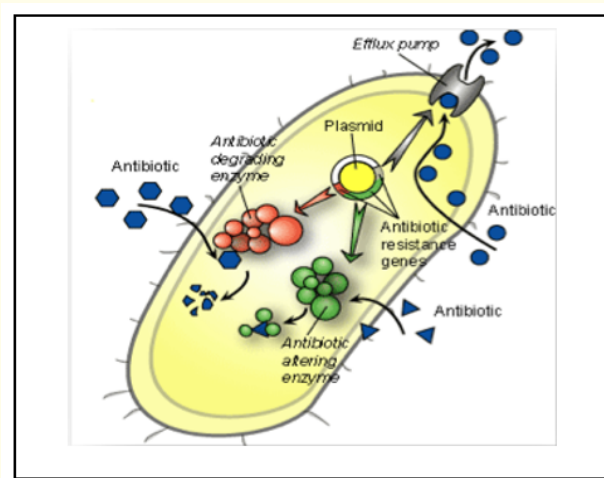
*Macrocooccus caseolyticus* is a gram positive cocci belonging to the family of *Staphylococcus caseolyticus* [23]. It is 1.1 - 2 µm in size, non-motile and non-spore bearing. It can occur alone, in pairs, short chains, or clusters [24]. Colonies are lightly convex, entire, butyrous, glistening and opaque. Colonies grow up to 3 - 4 mm in diameter on P agar and have weak to no anaerobic growth. They show growth in 10% NaCl and are non-haemolytic but may produce partial hemolysis (greening) of horse blood. Optimum growth temperature is 35°C. They are isolated mainly from raw cow milk and dairy products (surface cheese bacteria), reptile saliva (Komodo dragon), cetacean, sheep and goat milk and meat products. Furthermore, they are susceptible to Novobiocin (1.6 µg) [25].

### Antibiotic sensitivity

Antibiotic sensitivity testing is a laboratory method for determining the susceptibility of organisms to antibiotic therapy. After the infecting organism has been recovered from a clinical specimen, it is cultured and tested against a panel of antibiotic drugs (the specific panel is determined by whether the organism is Gram positive and Gram negative). If the growth of the organism is inhibited by the action of the drug, it is reported as sensitive to that antibiotic. If the organism continues to grow in presence of the drug, it is reported as resistant to that drug [26].

### Antibiotic resistance

Antibiotic resistance is the ability of certain strains of microorganisms to develop mechanisms to survive in the presence of antibiotics. Antibiotic resistant bacteria are bacteria that cannot be fully inhibited or killed by an antibiotic, despite being previously susceptible to that drug. Bacteria become resistant to antibiotics by adapting their structure or function in some way that prevents them from being killed by the antibiotic. This mechanism could happen in several ways shown in figure 6:



**Figure 6:** The nucleic acid synthesis inhibitor antibiotics. <http://www.nature.com/nrmicro/journal/v8/n6/full/nrmicro2333.html>.



- Bacteria can neutralize the antibiotic before it has an effect,
- Bacteria may be able to pump the antibiotic out of the cell,
- Bacteria may be able to alter the site (receptor) where the antibiotic normally works,
- Bacteria can mutate and transfer genetic material that codes for resistance to other bacteria [27].

### **Aim of the Study**

This *in vitro* study is undertaken to investigate the effect of twelve common antibiotics and tested alone and in combination against isolated environmental bacteria. These bacteria are isolated from the aerosol of Al Marj city, which is located in the eastern of Benghazi city (100 kilometers far). There isn't any research related to this subject from the past. These bacteria may be the key factors in the incidence of ambient air pollution in Al Marj city.

### **Materials and Methods**

#### **Materials**

##### **The equipment and its sources**

- Device counting colonies Model 00352 BIHJA from Stuart Scientific Company, Germany.
- Filter papers (3 MM) and laboratory waxing membranes from Whatman International, United Kingdom.
- The machine used in definition of bacterial strains (BD Phoenix 100) from the United States of America.
- Steam sterilizer (Autoclave) model HL 42AE, from Hirayama Manufacturing Corporation, Japan.
- Ordinary optical microscope from Japanese Olympus company.
- Incubator from British Gelinkam Company, UK.
- Glassware (Pyrex) from Pyrex Company, UK.
- The delicate balance from Sartarius Laboratory Company, Germany.
- Sterile petri dishes and sterile bags from Sterilin Company, the United Kingdom.
- Device counting colonies (colony counter), Model 3328, from American Optical Company.
- Cotton from Smith and Nephew Company, the United Kingdom.
- Sterile gloves from Bromed in the United States of America.
- Insulation rings (Loops) from Cultiplast Company in Italy.
- Microscope with Camera from Japanese Olympus Company.

### The antibiotics discs used

The antibiotics discs (content per disc) used in the study are: AMC: Amoxycillin/Clavulanic acid (30 µg); Ak: Amikacin (30 µg); C: Chloramphenicol (30 µg); FOX: Cefoxitin (30 µg); Cip: Ciprofloxacin (5 µg); CT: Colistin sulphate (10 µg); E: Erythromycin (15µg); CN: Gentamicin (10 µg); Ox: Oxacillin (1 µg); CL: Cephalexin (30 µg); TOB: Tobramycin (10 µg) and AMP: Ampicillin (10 µg) All antibiotic discs were purchased from Oxoid Ltd., Basingstoke Hampshire Company, England and shown in figure 7.



**Figure 7:** The antibiotic disks.

### General methods

#### Sources of experimental strains

The strains which ran in this experimental work were gifted from faculty members at Al Marj University (Personal Communication).

#### Sterile working practices

Sterilization of the materials necessary in practical experiments, including all nutrient agar and materials through the sterile steam (autoclave) in pressure 15 p.s.i. for 15 minutes, unless the materials or devices are sensitive to the impact of humid heat. Sterilized plastic dishes and other tools sensitive to heat were left for 24 hours in 70% alcohol solution then washed thoroughly with sterile water. Neck bottles and nozzles were sterilized by direct flame before and after use. The culture dishes were sterilized after molding nutrient media by passing the flame on the surface of the dissolved agar.

#### Incubation

The incubation period of the microorganisms are the most basic steps in laboratory applications for micro-organisms in view of the importance of this step in the growth process. In this study, the incubated dishes contained bacterial spores at a temperature of 37°C for 48 hours in the dark. The incubator shown in figure 8 was used to prevent the photoinactivation by light.





**Figure 8:** The incubator.

### **Preservation of bacterial cultures**

Bacterial cultures were saved in the radiator in the form of wax dishes. These cultures remained in the cold environmental conditions for a period of time. This prevents excessive breeding in developing shelf (stock culture). After some time, the sub culturing plates were placed in order to activate the bacterial cells and increase the vitality.

### **Purification of isolated bacteria**

The process of purification of bacterial isolates obtained from environment nutritious agar (nutrient agar) used the streaking method for plates in order to increase the vitality and then lapped in 37°C for 48 hours.

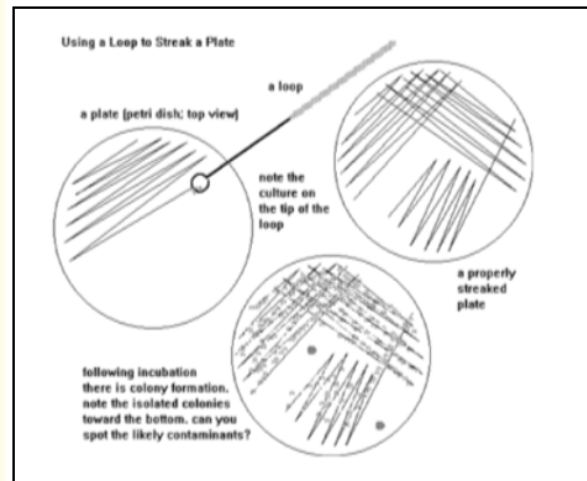
### **Sub culturing of isolated bacteria**

Sub-culturing is the aseptic transfer of micro-organisms from a culture to fresh medium. The freshly inoculated medium is then incubated at the temperature appropriate for growing the organism. There are four sub-culturing procedures with which are of importance:

1. Solid to solid: The transfer of bacteria or fungi from an agar slope or plate culture to an agar plate;
2. Solid to liquid: The transfer of bacteria or fungi from an agar slope or plate culture to a broth;
3. Liquid to solid: The transfer of bacteria or fungi from a broth culture to an agar slope or plate;
4. Liquid to liquid: The transfer of bacteria or fungi from a broth culture to a broth.

Containers of culture media to be inoculated must be labelled with initials, date and name of organism. To prevent possible confusion, plates are marked on the underside while tubes and bottles must be labelled on the side. Lids are not labelled. Cell lines and microorganisms cannot be held in culture indefinitely due to the gradual rise in toxic metabolites, use of nutrients and increase in cell number due to

growth. Subculture is therefore used to produce a new culture with a lower density of cells than the originating culture. Fresh nutrients and no toxic metabolites allowing continued growth of the cells without risk of cell death. Typically, subculture is from a culture of a certain volume into fresh growth medium of equal volume and allows long term maintenance of the cell line. Subculture into a larger volume of growth medium is used when wanting to increase the number of cells by using the streaking method. This method can be used in an industrial process or scientific experiment. Figure 9 shows the streaking method on nutrient and blood agar plate.



**Figure 9:** The procedure of streaking method on agar plate. <https://www.google.com.ly/search>.

In this study the isolated bacteria were sub cultured on nutrient agar and blood agar by using streaking method on the plates.

### Preparation of slides

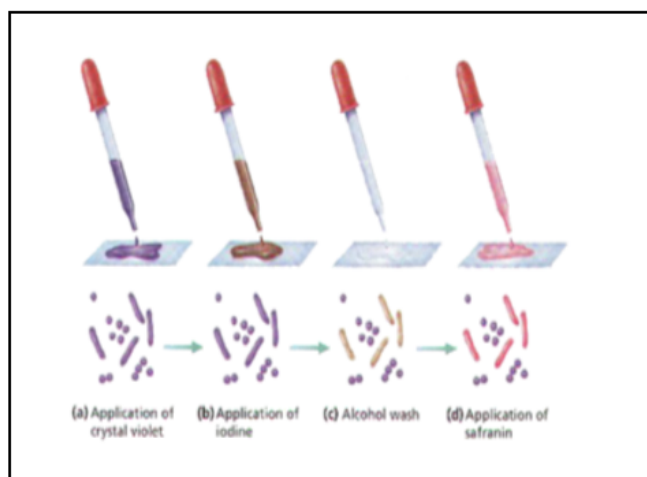
The bacteria were stained with Gram stain and differential by using dyes to differentiate between different groups of bacteria. One of these differential dyes are Gram stains. Staining bacteria in this way is one of the most important steps in the study of the properties of bacteria during the definition process. The four basic steps of the Gram Stain are:

1. Application of the primary stain Crystal Violet to a heat-fixed smear of bacterial culture. Crystal Violet dissociates in aqueous solutions into Crystal Violet and  $\text{Cl}^-$  ions. These two ions then penetrate through the cell wall and cell membrane of both Gram positive and Gram negative cells. The Crystal Violet + ions later interact with negatively charged bacterial components and stains the bacterial cells purple.
2. Addition of Gram's Iodine, which acts as a mordant and a trapping agent. A mordant is a substance that increases the affinity of the cell wall for a stain by binding to the primary stain, thus forming an insoluble complex which gets trapped in the cell wall. In the Gram stain reaction, the crystal violet and iodine form an insoluble complex (Crystal Violet-Iodine) which serves to turn the smear a dark purple color. At this stage, all cells will turn purple.
3. Decolorization with 95% ethyl alcohol. Alcohol or acetone dissolves the lipid outer membrane of Gram negative bacteria, thus leaving the peptidoglycan layer exposed and increases the porosity of the cell wall. The Crystal Violet-Iodine complex is then

washed away from the thin peptidoglycan layer, leaving Gram negative bacteria colorless. On the other hand, alcohol has a dehydrating effect on the cell walls of Gram positive bacteria which causes the pores of the cell wall to shrink. The CV-I complex gets tightly bound into the multi-layered, highly cross-linked Gram positive cell wall thus staining the cells purple.

The decolorization step must be performed carefully, otherwise over decolorization may occur. This step is critical and must be timed correctly otherwise the crystal violet stain will be removed from the Gram-positive cells. If the decolorizing agent is applied on the cell for too long, the Gram-positive organisms will appear Gram-negative. Under decolorization occurs when the alcohol is not left on long enough to wash out the Crystal Violet-Iodine complex from the Gram negative cells, resulting in Gram negative bacteria to appear Gram positive.

4. Counterstain with Safranin. The decolorized Gram negative cells can be rendered visible with a suitable counterstain, which is usually positively charged safranin, staining them pink. Pink colour which adheres to the Gram positive bacteria is masked by the purple of the crystal violet. Occasionally, basic fuschin is sometimes used instead of safranin, as shown in the figure 10.



**Figure 10:** The procedure of Gram stain. <http://biology.clc.uc.edu/fankhauser/labs/microbiology/Microscopy.html>.

The slide with safranin stain was washed well and slowly dried by filter paper. The stained slide was magnified with the help of the objective lens microprocessor 10x. A drop of immersion oil was added on the bacterial membrane formed after. It used the oily lens in order to get the form of bacterial dye. Gram positive bacteria have a thick mesh-like cell wall, which is made up of peptidoglycan (50 - 90% of cell wall), staining purple. Peptidoglycan is mainly a polysaccharide composed of two subunits called N-acetyl glucosamine and N-acetyl muramic acid. As adjacent layers of peptidoglycan are formed, they are cross linked by short chains of peptides by means of a transpeptidase enzyme, resulting in the shape and rigidity of the cell wall. The thick peptidoglycan layer of Gram positive organisms allows these organisms to retain the crystal violet-iodine complex and stains the cells purple. Lipoteichoic acid is another major constituent of the cell wall of Gram positive bacteria which is embedded in the peptidoglycan layer. It consists of teichoic acids, which are long chains of ribitol phosphate anchored to the lipid bilayer via a glyceride. It acts as regulator of autolytic wall enzymes like muramidases, bacterial enzymes located in the cell wall that cause disintegration of the cell following injury or death, as shown in figure 11.

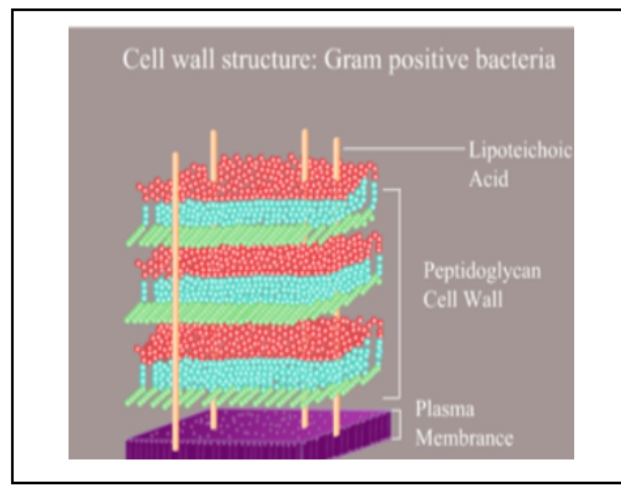


Figure 11: The cell wall structure of Gram positive bacteria. <http://amrita.vlab.co.in/?sub=3&brch=73&sim=208&cnt=1>.

The photographs of isolated bacteria slides after Gram staining were taken by microscope with associated camera (Figure 12).



Figure 12: The microscope with camera.

### The technique (The disk diffusion method)

One of the most widely used methods to determine the susceptibility of microorganisms to antimicrobial agents is the disc agar-diffusion method. The basis of this method is dependent upon the inhibition of reproduction of a microorganism on the surface of a solid medium by an antimicrobial agent, which diffuses into the medium from a filter paper disc. Thus, for an organism which is truly sensitive (susceptible) to an antimicrobial agent, a zone of inhibition around the disc impregnated with the agent should be seen. Beyond this zone, is an unaffected area of normal growth (lawn) of the organism.

Different microbial species and strains have different degrees of susceptibility to different chemotherapeutic agents. Moreover, the susceptibility of a microorganism can change with time, even during therapy with specific drug. Thus, a physician must know the sensitivities of the pathogen before treatment can be started. However, physicians often cannot wait for sensitivity tests and must begin treatment based on their estimation of the pathogen most likely causing the illness. Several tests can be used to indicate which chemotherapeutic agent is most likely to combat a specific pathogen. Tests are necessary only when susceptibility is not predictable or when problems with antibiotic resistance arise [28].

In Kirby-Bauer testing, bacteria are placed on a plate of solid growth medium and wafers of antibiotics (white disks shown) are added to the plate. After allowing the bacteria to grow overnight, areas of clear media surrounding the disks indicate that the antibiotic inhibited bacterial growth. The concentration of antibiotic that diffuses into the media decreases with increasing distance from the source. Therefore, the more sensitive the bacteria are to a given antibiotic, the larger the clear bacteria-free zone that forms around the disk containing that antibiotic [29].

In a Kirby-Bauer test, the size of the zone of inhibition indicates the degree of sensitivity of bacteria to a drug. In general, a larger area of bacteria-free media surrounding an antibiotic disk means the bacteria are more sensitive to the drug the disk contains. KB tests are performed under standard conditions, so the minimum inhibitory concentration for a given antibiotic can be calculated by comparing the size of zone of inhibition observed to known values. Clinicians use KB test results to choose antibiotics appropriate for the specific bacteria causing a patient's infection. Using specifically-targeted antibiotics helps decrease the incidence of antibiotic-resistant bacteria. Grabow and others reviewed the public health implications of drug-resistant coliforms in water supplies, and suggested that the prevalence of these drug-resistant bacteria requires re-evaluation of water quality standards as well as more advanced purification of sewage prior to discharge into the environment [30].

However, the degree of inhibition necessary to make each antibiotic effective during use is unique, hence each resistance zone size cutoff is specific to the antibiotic. Accurate measurement of zone diameter is necessary to properly interpret this test.

### Steps for disk diffusion method

1. *Bacillus coagulans*, *Brevibacillus brevis*, *Enterococcus raffinosus* and *Macrocooccus caseolyticus* were cultured from 12 to 18 hour. The cultured bacteria were plated on solidified blood agar and nutrient agar plates using sterile cotton swabs.
2. The antibiotic disc dispenser was used to dispense four antibiotic discs to each plate by following the following protocol:
  - a. The sterile cotton swab was dipped into the culture to be tested. The swab covered the entire surface of each of the solidified agar plates, such that a confluent lawn of growth would result if nothing more were to be done on the plates. The plates were left to dry at room temperature for several minutes.
  - b. With the disc dispensers, four antibiotic discs were applied to each plate to be sure that all twelve antibiotics were represented on the plates. The instructor demonstrated the use of the dispenser.
  - c. The plates were incubated for 1 - 2 days at 37°C.

### Antibiotic susceptibility

Antibiotic susceptibility of the isolates was tested by disc diffusion method with an inoculum of  $10^8$  cfu. The interpretive categories were defined according to the diameter of inhibition zone (Table 1) and by the list of the antibiotics that were used in the disk sensitivity test and their zones of clearing.

Antibiotic and disc identifier	Disc potency	Inhibition zone diameter to nearest (mm)		
		Resistant	Intermediate	Susceptible
Ampicillin (AM10)	10 µg	20	21- 28	29
Chloramphenicol (C)	30 µg	12 or less	13 - 17	18 or more
Ciprofloxacin (CIP)	5 µg	15 or less	16 - 20	21 or more
Colistin Sulphate (CT)	10 µg	8 or less	9 - 10	11 or more
Erythromycin (E)	15 µg	13 or less	14 - 17	18 or more
Gentamycin (CN)	10 µg	12	13 - 14	18
Oxacillin (OX)	1 µg	13 or less	13	13 or more
Tobramycin (TOB)	10 µg	12	13 - 14	15
Cephalexin (CL)	30 µg	14	15 - 17	18
Amikacin (AK)	30 µg	14 or less	15 - 16	20 - 26
Amoxicillin Clavlonic acid (AMC)	30 µg	19 or less	14 - 17	20 or more
Cefoxitin (FOX)	30 µg	21 or less	-	22 or more

**Table 1:** List of the antibiotics that were used in the disk sensitivity test and their zones of clearing.

The zone of clearing varies depending upon the antibiotic and its chemical properties. The medical effectiveness of antibiotics in patients has been correlated with a certain zone size and this is used to decide whether an antibiotic will be useful for treating a test pathogen. The size of the inhibition zones on the plates were measured with a ruler with millimeter (mm) divisions. The diameters of inhibition zones were measured in (mm) and compared to the standard measurements of inhibition zones in table 1:

1. With the ruler, measure the zones of inhibition in millimeters from the underside of the plate. Measure the entire diameter of the zone, including the disc.
2. Interpret your findings with the aid of the table below.
3. Compare the patterns of susceptibility and resistance with respect to gram reaction, potential pathogenicity and natural habitat of each organism.

Susceptible, intermediate or resistant interpretations of zone diameter measurements are reported and defined as follows:

1. Susceptible (S): The “susceptible” category implies that isolates are inhibited by the usually achievable concentrations of antimicrobial agent when the recommended dosage is used for the site of infection.
2. Intermediate (I): The “intermediate” category includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels, which response rates may be lower than susceptible isolates. The intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated (e.g. quinolones and β-lactams in urine) or when a higher than normal dosage of a drug can be used (e.g. β-lactams). This category also includes a buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations, especially for drugs with narrow pharmacotoxicity margins.
3. Resistant (R): The “resistant” category implies that isolates are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules, and/or that demonstrate zone diameters that fall in the range where specific microbial



resistance mechanisms (e.g. beta-lactamases) are likely, and clinical efficacy of the agent against the isolate has not been reliably shown in treatment studies.

### Antibiotic combination

Antibiotic synergism occurs when the effects of a combination of antibiotics is greater than the sum of the effects of the individual antibiotics, like amoxicillin/clavulanic acid, a combination antibiotic. Amoxicillin is an analog of ampicillin, derived from the basic penicillin nucleus. Potassium clavulanate is a  $\beta$ -lactamase inhibitor. In combination, these drugs are more effective than when used alone.

Antibiotic antagonism occurs when one antibiotic, usually the one with the least effect, interferes with the effect of another antibiotic. In figure 13, the combination antibiotic disks, containing augmentin antibiotic, used in this study are manufactured from Oxoid company. Combination therapy with two or more antibiotics is used in special cases to prevent the emergence of resistant strains, treat emergency cases during the period when an etiological diagnosis is still in progress, and to utilize antibiotic synergism.



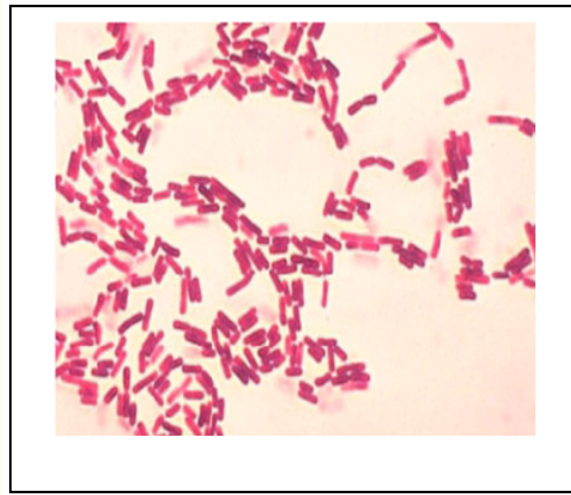
**Figure 13:** The combination antibiotic disk (Augmentin antibiotic).

### Results

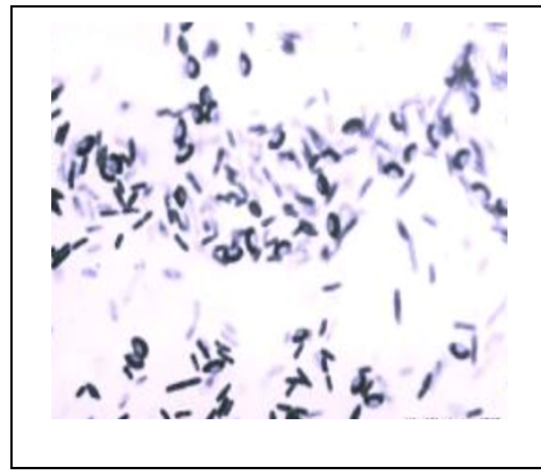
This applied study was conducted to investigate the effect of twelve common antibiotics when tested alone and in combination with amoxicillin/clavulanic acid antibiotic against isolated bacteria. The bacteria studied were *Bacillus coagulans*, *Brevibacillus brevis*, *Enterococcus raffinosus* and *Macrocooccus caseolyticus*. These bacteria were isolated from the atmosphere (Atmospheric bacterial load) of Al Marj city during the winter period from December 2012 to March 2013. The antibiotic discs used in this study were amoxicillin/clavulanic acid, amikacin, chloramphenicol, ceftiofur, ciprofloxacin, colistin sulphate, erythromycin, gentamicin, oxacillin, cephalixin, tobramycin, and ampicillin.

### The results of gram staining of isolated bacteria

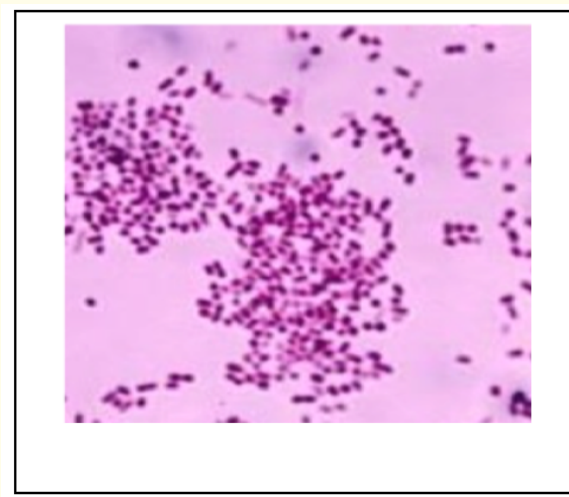
The isolated bacteria were differentiated using the Gram stain method. The photographs of the microscope slides were taken with a camera to find a clear picture of the Gram stained slides. Figure 14-17 show these images. All the bacteria in this study were Gram positive bacteria.



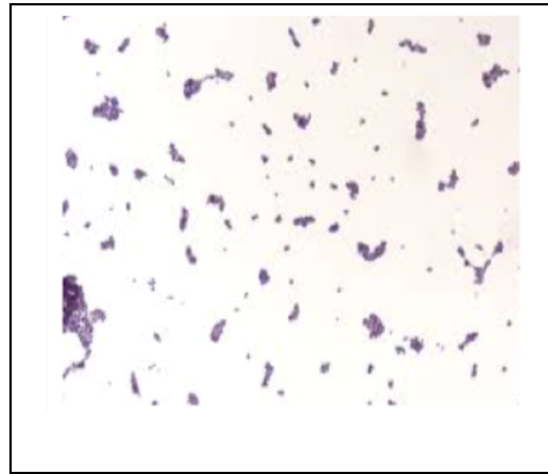
**Figure 14:** The gram stained *Bacillus coagulans* bacteria.



**Figure 15:** The gram stained *Brevibacillus brevis* bacteria.



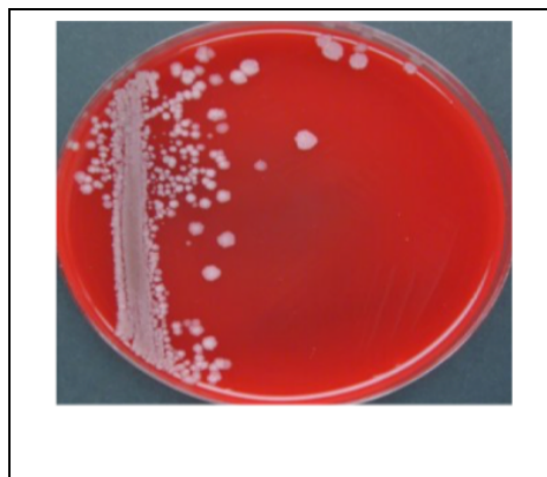
**Figure 16:** The gram stained *Enterococcus raffinosus* bacteria.



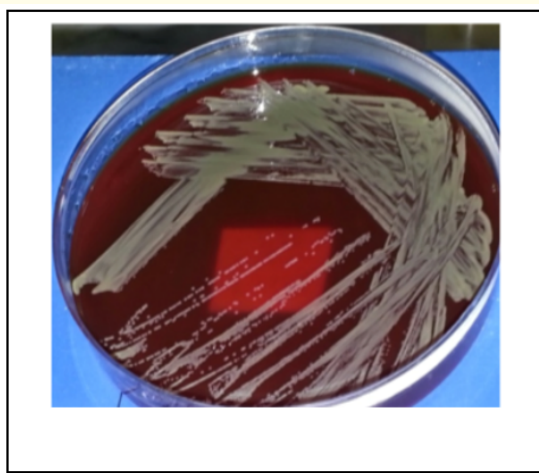
**Figure 17:** The gram stained *Macrocooccus caseolyticus* bacteria.

#### The results of sub culturing of isolated bacteria

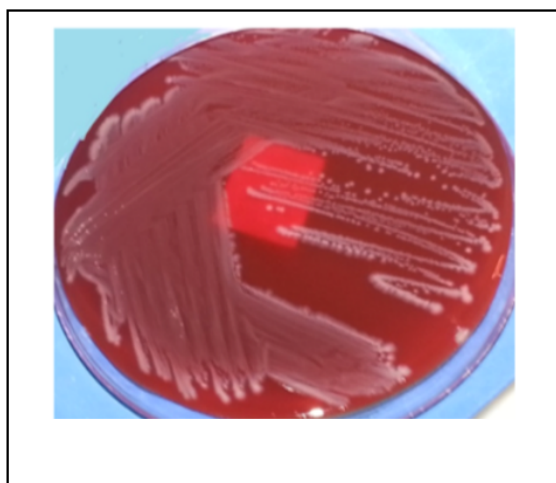
In the laboratory, micro-organisms are usually grown or cultured in liquid medium (broth) or on solid medium (agar plates or slopes). Growth of bacteria appears as cloudiness or turbidity in the broth, although sometimes bacteria grow as a layer on the surface of the broth or at the bottom of the culture tube. The growth on plates depends on how the plate has been inoculated. The isolated bacteria are sub-cultured on blood agar using the streaking method. Figure 18-21 show the growth of isolated bacteria on blood agar plate in this study.



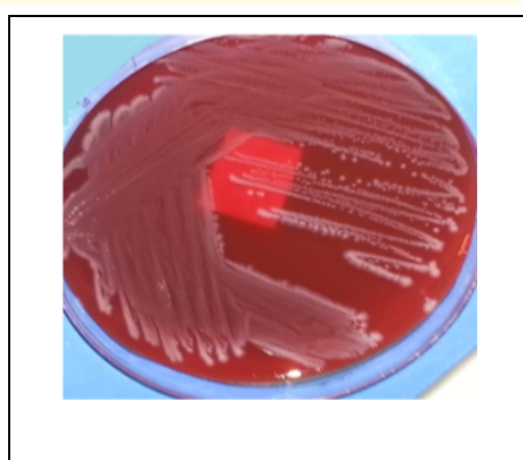
**Figure 18:** The growth of *Bacillus coagulans* bacteria on blood agar plate.



**Figure 19:** The growth of *Brevibacillus brevis* bacteria on blood agar plate.



**Figure 20:** The growth of *Enterococcus raffinosus* bacteria on blood agar plate.



**Figure 21:** The growth of *Macrocooccus caseolyticus* bacteria on blood agar plate.

### The antibacterial susceptibility of isolated bacteria

The antibiotics used in this study to conduct the disk diffusion method (Kirby Bauer test) were shown in colored disks. The ampicillin was shown in brown color, chloramphenicol in light green, ciprofloxacin in blue, colistin sulphate in yellow, erythromycin in white, gentamycin in dark blue, oxacillin in red, tobramycin in orange, cephalaxin in gray, amikacin in violet, amoxicillin/clavulanic acid in black, and cefoxitin in green. Their effects on four environmental bacteria are shown in figures 22, 24, 26 and 28.

### The susceptibility of *Bacillus coagulans*

The results of the disk sensitivity test for *Bacillus coagulans* showed sensitivity to amoxicillin/clavulanic acid with diameter zone 35 mm, tobramycin (15 mm), cephalaxin (18 mm), erythromycin (25 mm), chloramphenicol (29 mm), amikacin (26 mm), ciprofloxacin (26 mm), oxacillin (25 mm) and gentamycin (18 mm). *Bacillus coagulans* bacteria are intermediate to ampicillin with diameter zone 12 mm and cefoxitin (22 mm). *Bacillus coagulans* bacteria are resistant to colistin sulphate with diameter zone 7 mm as shown in the figure 22 and 23.

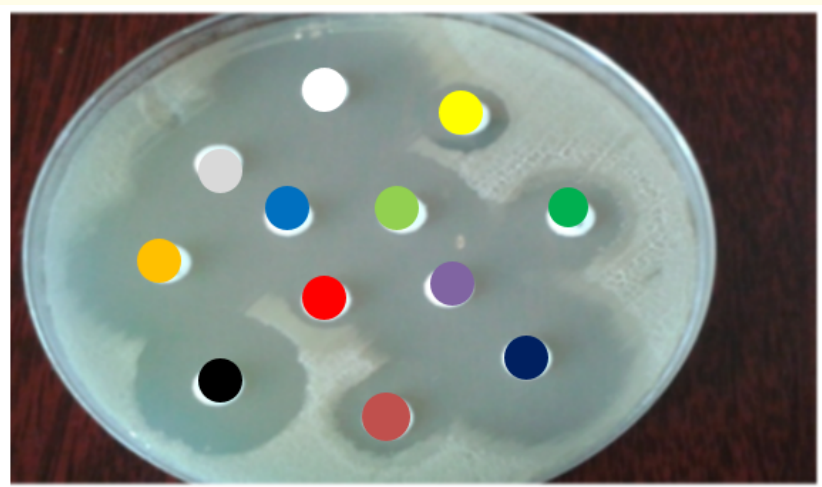


Figure 22: The disk sensitivity test for *Bacillus coagulans* bacteria.

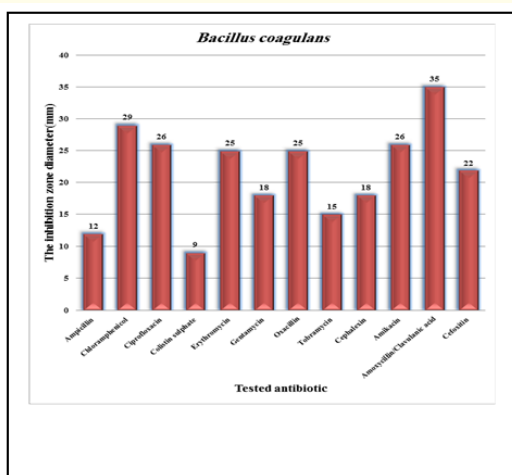


Figure 23: The susceptibility profiles of *Bacillus coagulans* bacteria.

### The susceptibility of *Brevibacillus brevis*

The results of the disk sensitivity test for *Brevibacillus brevis* bacteria showed sensitivity to amoxicillin/clavulanic acid with diameter zone 34 mm, chloramphenicol (27 mm), ciprofloxacin (28 mm), amikacin (24 mm), oxacillin (25 mm), tobramycin (15 mm), gentamycin (22 mm) and ampicillin (14 mm). The *Brevibacillus brevis* isolated bacteria are resistant to colistin sulphate with diameter zone 5 mm, cephalaxin (14 mm), erythromycin (12 mm) and ceftioxin (23 mm), as shown in figure 24 and 25.

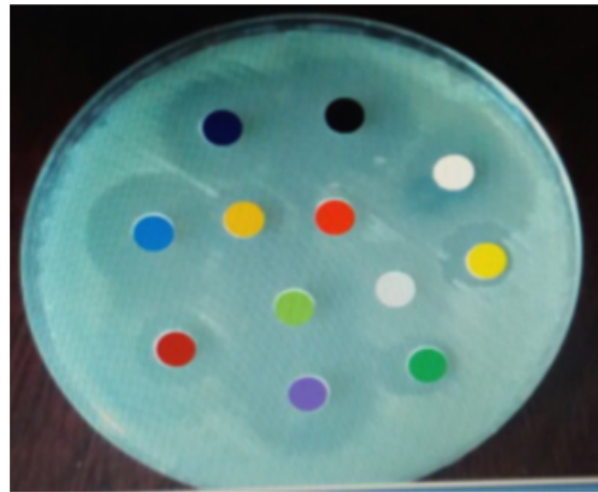


Figure 24: The disk sensitivity test for *Brevibacillus brevis* bacteria.

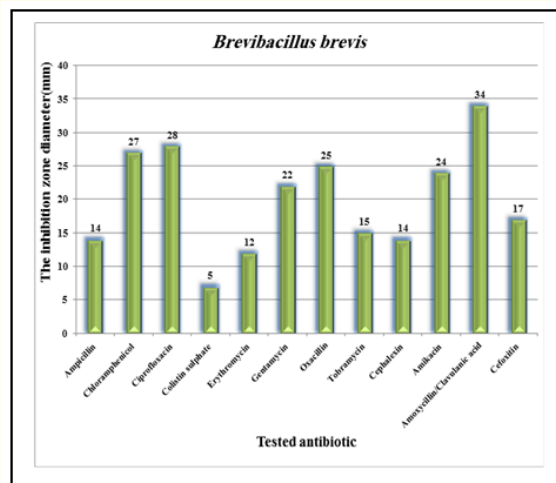


Figure 25: The susceptibility profiles of *Brevibacillus brevis* bacteria.



### The susceptibility of *Enterococcus raffinosus*

In this study, the current environmental *Enterococcus raffinosus* bacteria was susceptible or sensitive to erythromycin with diameter zone 23 mm, ciprofloxacin (27 mm), chloramphenicol (28 mm), tobramycin (15 mm), amikacin (25 mm), cefoxitin (28 mm), ampicillin (29 mm), amoxicillin/clavulanic acid (33 mm), cephalixin (18 mm), oxacillin (25 mm), and gentamycin (18 mm). *Enterococcus raffinosus* bacteria was resistant to Colistin sulphate with diameter zone 8 mm as shown in figure 26 and 27.

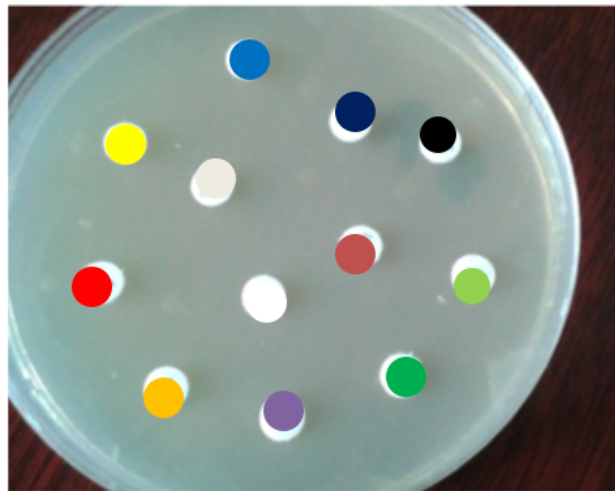


Figure 26: The disk sensitivity test for *Enterococcus raffinosus* bacteria.

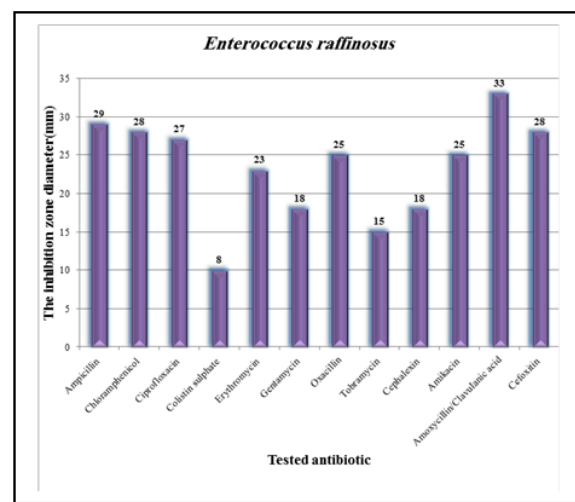


Figure 27: The susceptibility profiles of *Enterococcus raffinosus* bacteria.

### The susceptibility of *Macrocooccus caseolyticus*

The results of the disk sensitivity test for *Macrocooccus caseolyticus* bacteria showed sensitivity to erythromycin with diameter zone 24 mm, ciprofloxacin (25 mm), chloramphenicol (28 mm), tobramycin (15 mm), amikacin (25 mm) and gentamycin (18 mm). The *Macrocooccus caseolyticus* bacteria are resistant to amoxicillin/clavulanic acid with diameter zone 11 mm, cephalixin (3 mm), colistin sulphate (6 mm), oxacillin (3 mm), ampicillin (2 mm) and ceftoxitin (11 mm), as shown in figure 28 and 29.

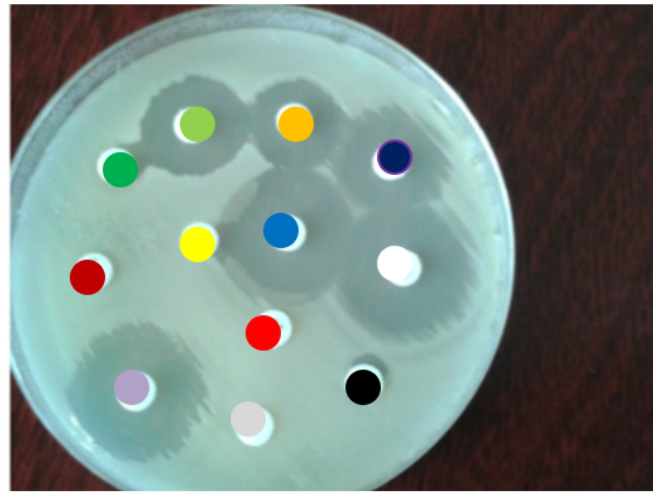


Figure 28: The disk sensitivity test for *Macrocooccus caseolyticus* bacteria.

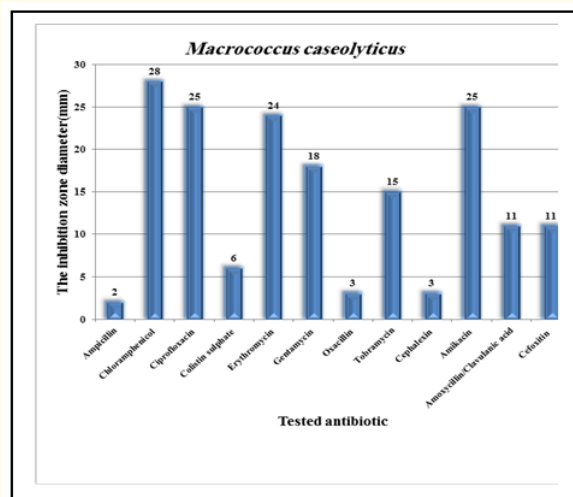


Figure 29: The susceptibility profiles of *Macrocooccus caseolyticus* bacteria.

And these results of Zones of clearing for various antibiotics of the environmental bacteria are listed on the table 2 and figure 30.

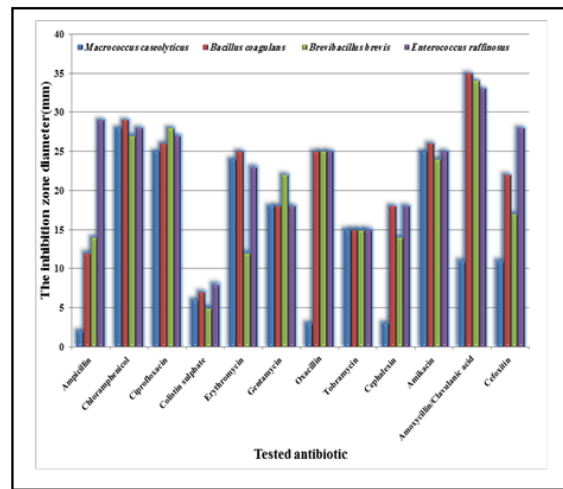


Figure 30: The comparison of environmental bacterial isolates to different antibiotic.

Antibiotic and disc identifier	Disc potency	Macrocooccus caseolyticus	Bacillus coagulans	Brevibacillus brevis	Enterococcus raffinosus
Ampicillin (AM10)	10 µg	Resistant ~ 20 mm	Intermediate ~ 21 mm	Intermediate ~ 21 mm	Susceptible ~ 29 mm
Chloramphenicol (C)	30 µg	Susceptible ~ 28 mm	Susceptible ~ 29 mm	Susceptible ~ 27 mm	Susceptible ~ 28 mm
Ciprofloxacin (CIP)	5 µg	Susceptible ~ 25 mm	Susceptible ~ 26 mm	Susceptible ~ 28 mm	Susceptible ~ 27 mm
Colistin Sulphate (CT)	10 µg	Resistant ~ 6 mm	Resistant ~ 7 mm	Resistant ~ 5 mm	Resistant ~ 8 mm
Erythromycin (E)	15 µg	Susceptible ~ 24 mm	Susceptible ~ 25 mm	Resistant ~ 12 mm	Susceptible ~ 23 mm
Gentamycin (CN)	10 µg	Susceptible ~ 18 mm	Susceptible ~ 18 mm	Intermediate ~ 13 mm	Susceptible ~ 18 mm
Oxacillin (OX)	1 µg	Resistant ~ 10 mm	Susceptible ~ 17 mm	Susceptible ~ 17 mm	Susceptible ~ 25 mm
Tobramycin (TOB)	10 µg	Susceptible ~ 15 mm	Susceptible ~ 15 mm	Intermediate ~ 13 mm	Susceptible ~ 15 mm
Cephalexin (CL)	30 µg	Resistant ~ 14 mm	Susceptible ~ 18 mm	Resistant ~ 14 mm	Susceptible ~ 18 mm
Amikacin (AK)	30 µg	Susceptible ~ 25 mm	Susceptible ~ 26 mm	Susceptible ~ 24 mm	Susceptible ~ 25 mm
Amoxicillin Clavulonic acid (AMC)	30 µg	Resistant ~ 19 mm	Susceptible ~ 35 mm	Susceptible ~ 34 mm	Susceptible ~ 33 mm
Cefoxitin (FOX)	30 µg	Resistant ~ 10 mm	Intermediate ~ 22 mm	Resistant ~ 17 mm	Susceptible ~ 28 mm

Table 2: The environmental bacteria and inhibition zones of antibiotics.

## Discussion

The widespread emergence of antibiotic resistance, particularly multidrug resistance among bacterial pathogens has become one of the most serious challenges in clinical therapy [31]. Environments containing antibiotic residues exert selection pressure and contribute to the appearance of resistant bacteria. In light of the potential health risk, many studies have focused on antibiotic-resistant bacteria from various ecosystems [32]. In the present study, the bacteria were isolated from the aerosol of Al Marj city, located east of Benghazi City, and their pattern of sensitivity and resistance various antibiotics was studied. Fortunately, Al Marj City is in the eastern part of Libya, which was not studied previously in terms of environmental microbiology. The bacteria of interest in this study were *Bacillus coagulans*, *Brevibacillus brevis*, *Enterococcus raffinosus* and *Macroccoccus caseolyticus*.

Polymyxins E (also known as colistin) were used in the treatment of Gram negative bacterial infections [8]. After binding to lipopolysaccharide (LPS) in the outer membrane of Gram negative bacteria, polymyxins disrupt both the outer and inner membranes. The hydrophobic tail is important in causing membrane damage, suggesting a detergent-like mode of action. Removal of the hydrophobic tail of polymyxin B yields polymyxin Nona peptide, which still binds to LPS, but no longer kills the bacterial cell. It does, however, increase the permeability of the bacterial cell wall to other antibiotics, indicating that it still causes some degree of membrane disorganization [33]. This study found the four Gram positive bacteria are resistant to colistin sulphate antibiotic, which may be due to the cationic (positively charged) polymyxin molecules attracting the negatively charged bacteria. The negative charge of bacteria is due to lipopolysaccharide (LPS) in the outer membrane and peptidoglycan (notably the teichoic acid). The antibiotic binds to the cell membrane, alters its structure and makes it more permeable. This disrupts osmotic balance causing leakage of cellular molecules, inhibition of respiration and increased water uptake leading to cell death. The antibiotic acts much like a cationic detergent and effects all membranes similarly, and therefore, toxic side effects are common. Little or no effect on Gram positive bacteria is seen since the cell wall is too thick to permit access to the membrane, and so, Gram positives are naturally resistant. The results of this study were in line with some previous studies, which similarly found that bacteria isolated from the environment (soil) were resistant to colistin sulphate and *Pseudomonas aeruginosa* was resistant to colistin sulphate, too [34].

Ciprofloxacin is a second generation fluoroquinolone antibiotic [35]. Its spectrum of activity includes most strains of bacterial pathogens responsible for respiratory, urinary tract, gastrointestinal, and abdominal infections, including Gram negative bacteria. It kills bacteria by interfering with the enzymes, primarily topoisomerases, causing DNA to rewind after being copied, stopping bacterial synthesis of DNA [36]. Quinolones, like ciprofloxacin, have no problem crossing the outer membrane. They easily diffuse through the peptidoglycan and the cytoplasmic membrane and rapidly reach their target, topoisomerases such as DNA gyrase. Quinolones have rapid bactericidal activity. The bacterial chromosome is supercoiled by the enzyme DNA gyrase. A fully supercoiled chromosome will be about 1 micron in diameter small enough to fit in the bacteria. The bacterial chromosome consists of a single circle of DNA. DNA is double stranded forming a left handed double helix. All topoisomerases can relax DNA but only gyrase can carry out DNA supercoiling, a process necessary to compact the bacterial chromosome which is 1000 times longer than the bacterial cell.

Topoisomerases are also involved in DNA replication, transcription, and recombination. The main quinolone target is the DNA gyrase, which is responsible for cutting one of the chromosomal DNA strands at the beginning of the supercoiling process. The nick is only introduced temporarily and later the two ends are joined back together (i.e. repaired). The quinolone molecule forms a stable complex with DNA gyrase, thereby inhibiting its activity and preventing the repair of DNA cuts.

The results of this study were in agreement with some previous studies, which similarly found that *Edwardseilla tarda* was susceptible to ciprofloxacin. *K. pneumoniae*, *E. coli*, *S. aureus*, *P. aeruginosa* and *Acinetobacter* were sensitive to ciprofloxacin [37]. Similarly, *Proteus vulgaris* was also sensitive to ciprofloxacin [38]. However, the findings disagree with few other studies, which found that all strains were

resistant to ciprofloxacin except for *S. typhi* and *E. aerogenes*, which was intermediate for them [39]. According to one study, cephalosporin resistant *Escherichia coli* on fattening farms were resistant to fluoroquinolones [40], *Salmonella enterica* serotype typhimurium had reduced susceptibility to fluoroquinolones [41] and *Burkholderia* species were intrinsically resistant to ciprofloxacin [35].

The four Gram positive species in this study were highly sensitive to chloramphenicol antibiotic, which is very active against many Gram positive and Gram negative bacteria like *Chlamydia*, *Mycoplasma* and Rickettsiae. Chloramphenicol antibiotic is a relatively small molecule that easily enters Gram positive and Gram negative bacteria. Once inside, it targets the 50S ribosomal subunit where it inhibits the elongation step of protein synthesis. The results of this study were in agreement with some previous studies, which also found that *Micrococcus radiodurans* was susceptible to chloramphenicol [42], *Microbacterium oxydans* was resistant to chloramphenicol and a *Kocuria* species was sensitive to chloramphenicol [43]. The study disagreed with other studies which found that *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* were resistant to chloramphenicol [44], bacteria isolated from soil were resistant to chloramphenicol [33], *Salmonella enterica* serotype typhimurium was usually resistant to chloramphenicol, *Escherichia coli* and *Comamonas testosteroni* were resistant to chloramphenicol [45].

Gentamicin is an aminoglycoside antibiotic composed of a mixture of related gentamicin components and fractions and is used to treat many types of bacterial infections, particularly those caused by Gram negative organisms [46]. It is synthesized by *Micromonospora*, a genus of Gram positive bacteria widely present in the environment (water and soil) and is active against a wide range of human bacterial infections [47].

Amikacin is an aminoglycoside antibiotic used to treat various types of bacterial infections. Amikacin works by binding to the bacterial 30S ribosomal subunit, causing errors in mRNA reading and leaving the bacterium unable to synthesize essential proteins for growth.

Tobramycin is an aminoglycoside antibiotic derived from *Streptomyces tenebrarius* and is used to treat various types of bacterial infections, particularly Gram-negative infections. It is especially effective against species of *Pseudomonas* [48]. Tobramycin works by binding to a site on the bacterial 30S and 50S ribosome, preventing formation of the 70S complex. As a result, mRNA cannot be translated into protein and cell death ensues.

The gram positive species studied in this research were highly sensitive or susceptible to aminoglycoside antibiotics like amikacin, tobramycin and gentamicin because aminoglycosides are positively charged molecules that rapidly enter the negatively charged bacterial cell. The negative charge of bacteria is due to lipopolysaccharide (LPS) in the outer membrane and the peptidoglycan (notably the teichoic acid). The drugs cross the cytoplasmic membrane via bacterial respiratory enzymes involved in aerobic respiration. For this reason, bacteria without respiratory enzymes, like strict anaerobes or facultative anaerobes, are naturally resistant to aminoglycosides. However, the four gram positive strains studied in this paper were sensitive to aminoglycosides. The results of this study were similar to some previous studies which also found that *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were susceptible to amikacin [49], *Salmonella Typhimurium* was susceptible to gentamicin [50] and *K. pneumoniae*, *E. coli*, *S. aureus*, *Pseudomonas aeruginosa* and *Acinetobacter* were sensitive to gentamicin [51]. The results did disagree with few other studies which found that bacteria isolated from soil were resistant to amikacin and gentamicin [31], *Proteus vulgaris* was resistant to gentamicin [36], *Pseudomonas aeruginosa* was resistant to tobramycin [33] and *K. pneumoniae*, *E. coli*, *S. aureus*, *Pseudomonas aeruginosa*, *Acinetobacter* and all strains used in the study were resistant to amikacin [35].

Erythromycin is a macrolide antibiotic that has an antimicrobial spectrum similar to or slightly wider than that of penicillin. Erythromycin is produced from a strain of the *Actinomycete*, *Saccharopolyspora* and *erythraea*. Erythromycin displays bacteriostatic activity, inhibiting the growth of bacteria, especially at higher concentrations [21], but the mechanism is not fully understood. By binding to the 50s subunit of the bacterial 70s rRNA complex, protein synthesis is blocked and proteins essential for structure and function are not formed

[58]. Erythromycin interferes with aminoacyl translocation, preventing the transfer of the tRNA bound at the A site of the rRNA complex to the P site of the rRNA complex. Without this translocation, the A site remains occupied and, thus, the addition of an incoming tRNA and its attached amino acid to the nascent polypeptide chain is inhibited. This interferes with the production of functionally useful proteins, which is the basis of this antimicrobial action.

The Gram positive bacteria in this study were sensitive to erythromycin antibiotic with the exception of *Brevibacillus brevis* bacteria, which was resistant to erythromycin. Erythromycin is a macrolide antibiotic. Macrolides are bacteriostatic. Their spectrum of activity is limited to Gram positive cocci such as Streptococci and Staphylococci. These antibiotics are also active against anaerobes. The drugs enter a gram positive cell without easily since peptidoglycan and the cytoplasmic membrane do not act as a barrier for the diffusion of these molecules. Once inside the cell, erythromycin targets the 50S ribosomal subunit in the cytoplasm. Macrolide antibiotics are structurally distinct but have a similar modes of action by binding the 50S ribosomal subunit. During translation, it blocks the initiation step, elongation step or peptide release step of protein synthesis. Unfinished or toxic protein is therefore released and is not functional.

Two principal mechanisms of resistance to macrolide antibiotics have been identified in Gram positive bacteria. Erythromycin resistant methylase is encoded by *erm* genes. Resultant structural changes to rRNA prevent macrolide binding and allow synthesis of bacterial proteins to continue. Presence of the *erm* gene results in high-level resistance. Modification of the mechanism whereby antibiotics are eliminated from the bacteria also brings about resistance. Bacteria carrying the gene encoding macrolide efflux (i.e. the *mef E* gene) display relatively low-level resistance. Other resistance mechanisms involve stimulation of enzymatic degradation but appear not to be clinically significant [52]. The results of this study agreed with some previous studies which similarly found that *Micrococcus radiodurans* [40], *Aeromonas* spp, *S. typhi*, and *E. aerogenes* were sensitive to erythromycin [37] and disagreed with few other studies which found that bacteria isolated from soil were resistant to erythromycin [31] *Proteus vulgaris* was resistant to erythromycin [36]. Further, bacteria from water, sediments and fish were collected from fish farm isolates and were screened for the presence of resistance genes against various antimicrobials, including erythromycin resistance [*mef A*] [53].

Ampicillin, oxacillin, cephalexin, cefoxitin and augmentin antibiotics are beta lactams which are mostly water soluble. Differences in cell wall composition (lipid composition) between different bacterial species partially accounts for their differential susceptibility to  $\beta$ -lactams. The target of the  $\beta$ -lactam antibiotics for all bacteria is the PBP (Penicillin Binding Protein) in the cytoplasmic membrane. In gram positive bacteria, there is no barrier to the entry of  $\beta$ -lactams antibiotics. The peptidoglycan layer allow the diffusion of small molecules. However,  $\beta$ -lactams have a difficult time crossing the membrane due to the high lipid content. However, since the target PBP's are found on the outer surface of the cytoplasmic membrane, they only have to cross the cell wall.

Ampicillin belongs to the penicillin group of beta lactam antibiotics and is able to penetrate Gram positive and some Gram negative bacteria. It differs from penicillin G or benzyl penicillin because it contains an amino group. That amino group helps the drug penetrate the outer membrane of Gram negative bacteria. Ampicillin acts as an irreversible inhibitor of the enzyme transpeptidase, which is a required enzyme for bacterial cell wall synthesis [54]. It inhibits the third and final stage of bacterial cell wall synthesis in binary fission and ultimately leads to cell lysis. Ampicillin has a broad spectrum of activity and has been used to treat bacterial infections in the genitourinary and lower respiratory tract [55]. Ampicillin has received FDA approval for its mechanism of action. Antibiotic resistance to ampicillin develops when bacteria develop an enzyme responsible for breaking down the beta lactam ring seen in ampicillin. This degrades the antibiotic surrounding the resistant bacteria, allowing the bacteria to survive.

In this study, it was found that *Macroccoccus caseolyticus* is resistant to ampicillin, *Bacillus coagulans* bacteria is intermediate to ampicillin, and *Enterococcus raffinosus* and *Brevibacillus brevis* are sensitive to ampicillin. The results of this study were in agreement with some previous studies which also found that a *Kocuria* sp. was sensitive to ampicillin [41] and disagree with other studies which found



that *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* [42], *Microbacterium oxydans* [50], *Salmonella enterica* [41], *Klebsiella Pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris* [36], *Comamonas testosterone* [43] and *Aeromonas hydrophila* were resistant to ampicillin [32].

Oxacillin is a narrow spectrum, beta lactam antibiotic of the penicillin class. Oxacillin is a penicillinase-resistant  $\beta$ -lactam [56] and exerts bactericidal activity via inhibition of bacterial cell wall synthesis by binding one or more of the penicillin binding proteins (PBPs). Oxacillin also exerts bacterial autolytic effects by inhibiting certain PBPs related to the activation of a bacterial autolytic process.

Oxacillin is similar in structure to methicillin and has replaced methicillin in clinical use. Since it is resistant to penicillinase enzymes, such as those produced by *Staphylococcus aureus*, it is widely used clinically in to treat penicillin resistant *Staphylococcus aureus*. However, with the introduction and widespread use of both oxacillin and methicillin, antibiotic resistant strains called oxacillin resistant *Staphylococcus aureus* (MRSA/ORSA) have become increasingly prevalent worldwide. In this study, all gram positive bacteria were found to be sensitive to oxacillin except *Macrocooccus caseolyticus*, which was resistant.

Amoxicillin/clavulanic acid is a combination antibiotic consisting of amoxicillin trihydrate, a  $\beta$ -lactam antibiotic similar to ampicillin, and potassium clavulanate, a  $\beta$ -lactamase inhibitor. This combination results in an antibiotic with an increased spectrum of activity and restored efficacy against amoxicillin resistant bacteria that produce  $\beta$ -lactamase. The combination was discovered around 1977 by British scientists working at Beecham, a pharmaceutical company. Clavulanic acid is produced by the fermentation of *Streptomyces clavuligerus*. It is structurally similar to the  $\beta$ -lactam ring of penicillins and possesses the ability to inactivate a wide variety of  $\beta$  lactamases by blocking the active sites of these enzymes. Clavulanic acid is particularly active against the clinically important plasmid mediated  $\beta$ -lactamases frequently responsible for transferred drug resistance to penicillins and cephalosporins [57]. However, when used alone, clavulanic acid has poor antibacterial activity. When in combination, it is hydrolyzed by bacterial enzymes, leaving the antibiotic to function and kill bacteria.

The two main mechanisms of resistance to amoxicillin/clavulanic acid combinations are inactivation by bacterial  $\beta$ -lactamases that are not themselves inhibited by clavulanic acid and also through alteration of PBPs, reducing the affinity of the antibacterial agent for the target. In this study, all gram positive bacteria were found to be sensitive to augmentin (amoxicillin/clavulanic acid) except *Macrocooccus caseolyticus* bacteria, which was resistant. The results of this study disagreed with some previous studies, which found that *Klebsiella Pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus vulgaris* were resistant to augmentin [36].

Cefoxitin is a cephamycin antibiotic, containing a  $\beta$ -lactam ring, and is often grouped with the second generation cephalosporins. Cefoxitin acts by interfering with cell wall synthesis and is therefore a bactericidal agent. Its activity may be hindered in the presence of some beta lactamases, including penicillinases and cephalosporinases, in Gram negative and Gram positive bacteria [58]. In this study, *Macrocooccus caseolyticus* and *Brevibacillus brevis* bacteria were resistant to cefoxitin, while *Bacillus coagulans* and *Enterococcus raffinosus* were sensitive to cefoxitin. Resistance to cefoxitin primarily occurs through hydrolysis by beta lactamases, alteration of penicillin binding proteins (PBPs), and decreased permeability to the drug. Another study found that *Aeromonas hydrophila* was resistant to cefoxitin and *Pseudomonas aeruginosa* was resistant to cefoxitin [32].

Cefalexin, or more commonly known as Cephalexin, is a first generation semi-synthetic cephalosporin antibiotic. Cephalexin, like Penicillin, is a beta lactam antibiotic. By binding to specific penicillin binding proteins (PBPs) located inside the bacterial cell wall, it inhibits the third and final stage of bacterial cell wall synthesis. Cell lysis is then mediated by bacterial cell wall autolytic enzymes such as autolysins. It is also possible that cephalexin interferes with an autolysin inhibitor [59]. Cephalexin antibiotic is bactericidal and has better activity against Gram positive bacteria compared to Gram negative bacteria.

*Enterococcus*-PBP's vary from other Gram positive bacteria and also have higher lipid content in the bacterial cell wall, providing a low level resistance to penicillins and first generation cephalosporins. The mechanism by which bacterial resistance to cephalexin occurs

is through destruction of the beta lactam ring by beta lactamases. An intact beta lactam ring is essential for antibacterial activity or by altered affinity of cephalosporins for their target site, the penicillin binding proteins. In this study *Macrocooccus caseolyticus* and *Brevibacillus brevis* bacteria were resistant to cephalixin, while *Bacillus coagulans* and *Enterococcus raffinosus* were sensitive to cephalixin.

The high prevalence of indigenous antibiotic resistant bacteria harboring diverse resistance traits could represent a potential health risk. Humans become infected with multi-drug resistant (MDR) environmental bacteria through consumption of contaminated water and vegetables. Antibiotic resistance genes might be transferred to pathogenic bacteria infecting humans particularly under the selection pressure of antibiotics through the SOS response [59].

Li and others demonstrated that even the administration of a single antibiotic or long term exposure of microorganisms to a high concentration of the antibiotic, can select for MDR strains. Acquisition of resistance genes through horizontal transfer facilitated by plasmids has been found to be ubiquitous in clinical pathogens [60].

Various agricultural and anthropogenic activities have led to a vast number of chemicals, including antibiotics, entering soil ecosystems and causing a major global concern because of their toxicity and threat to human life. Most of the known antibiotics are produced by actinomycetes and are commonly found in soils, compost and other environmental sources. The soil dwelling bacteria inhabiting an environment containing antibiotics develop diverse mechanisms to survive and resist the toxic antimicrobial compounds produced by their neighbors.

Given the increasing knowledge of environmental reservoirs of resistance, it should now be possible to have early signs of potential resistance mechanisms to new or old antibiotics and thus proactively combat clinical problems that may arise. It is incumbent on us to renew a concerted offensive that takes full advantage of the new understanding and technologies [60]. If not, the pre antibiotic era awaits our descendants.

## **Conclusion**

Through this field study, we conclude that the area is one not previously studied by researchers of environmental bacteriology. Al Marj City thrives on its agricultural activities, which has led to increased microbial activity and microbial contamination. The importance and value of antibiotics cannot be overestimated as humans are very dependent on them for the treatment of infectious disease. In addition to their use in the treatment of infectious diseases, antibiotics are critical to the success of advanced surgical procedures, including organ and prosthetic transplants.

Antibiotic resistance mechanisms are pandemic and create an enormous clinical and financial burden on health care systems worldwide. There are no simple solutions to the problem. Decisive actions that require significant commitment and enforcement are never popular, even if lives can be saved.

Fortunately, not all bacterial pathogens are resistant at once and in fact, many respond to empirical treatment with antimicrobial agents administered in the community. Given the many imponderables, the best that one can expect is that physicians and health care centers provide patients with environments that are resistance free, by taking enforcing more strict measures for antibiotic use. This must be backed up by efforts to prevent dumping of antibiotics into the environment through sewer systems. Complete destruction of antibiotics before disposal should be common practice to help prevent widespread bacterial resistance.

One of the major shifts in the relationship of microorganisms and antibiotics has been the increased incidence of antimicrobial resistant Gram positive pathogens. Treatment of serious Gram positive bacterial infections is complicated by this emergence of resistant pathogens and by increasingly complex patient populations who are susceptible to these pathogens. Researchers continue to develop new and effective antimicrobial agents to combat these resistant bacteria.

This phenomenon of high antibiotic resistance, as shown in this study, is important evidence of the direct exposure of antibiotics to humans and animals. The widespread use of antibiotics and their accumulation in different ecological niches cause development of antibiotic resistance in bacteria through the air, soil, and water. The study of resistance in the environmental bacteria helps predict future emergence and guide the development of strategies to counteract this resistance.

## **Bibliography**

1. Demain AL. "Small bugs, big business, the economic power of the microbes". *Biotechnology Advances* 18.6 (2000): 499-514.
2. Holten KB., *et al.* "Appropriate prescribing of oral beta-lactam antibiotics". *American Family Physician* 62.3 (2000): 611-620.
3. Elander RP. "Industrial production of beta-lactam antibiotics". *Applied Microbiology and Biotechnology* 61.5-6 (2003): 385-392.
4. Fisher JF., *et al.* "Bacterial Resistance to  $\beta$ -Lactam Antibiotics: Compelling Opportunism, Compelling Opportunity". *Chemical Reviews* 105.2 (2005): 395-424.
5. Kaiser G. "Protein synthesis inhibitors: macrolides mechanism of action animation. Classification of agents". *Pharmamotion* (2005): 19-52.
6. Tenson T., *et al.* "The Mechanism of Action of Macrolides, Lincosamides and Streptogramin B Reveals the Nascent Peptide Exit Path in the Ribosome". *Journal of Molecular Biology* 330.5 (2003): 1005-1014.
7. Falagas ME., *et al.* "Potential of old-generation antibiotics to address current need for new antibiotics". *Expert Review of Anti-Infective Therapy* 6.5 (2008): 593-600.
8. Jardetzky O. "Studies on the Mechanism of Action of Chloramphenicol - The Conformation of Chloramphenicol in Solution". *The Journal of Biological Chemistry* 238.7 (1963): 2498-2508.
9. Aminoglycosides at the US National Library of Medicine (2011).
10. Wilschanski M., *et al.* "Gentamicin Induced Correction of CFTR Function in Patients with Cystic Fibrosis and CFTR Stop Mutations". *New England Journal of Medicine* 349.15 (2003): 1433-1441.
11. Ryden R., *et al.* "The *In-vitro* activity of apramycin, a new aminocyclitol antibiotic". *Journal of Antimicrobial Chemotherapy* 3.6 (1977): 609-613.
12. Dewick PM. "Medicinal Natural Products: A Biosynthetic Approach, 3<sup>rd</sup> edition". *John Wiley and Sons* (2009).
13. Shakil S., *et al.* "Aminoglycosides versus bacteria - a description of the action, resistance mechanism, and nosocomial battleground". *Journal of Biomedical Science* 15.1 (2008): 5-14.
14. Aldred KT., *et al.* "Mechanism of Quinolone Action and Resistance". *Biochemistry* 53.10 (2014): 1565-1574.
15. Dixon RA., *et al.* "Polymyxin B and polymyxin B nonapeptide alter cytoplasmic membrane permeability in *Escherichia coli*". *Journal of Antimicrobial Chemotherapy* 18.5 (1986): 557-563.
16. Polymyxins at the US National Library of Medicine (2011).
17. Hammer BW. "Bacteriological studies on the coagulation of evaporated milk". *Iowa Agriculture and Home Economics Experiment Station Research Bulletin* 2.19 (1915): 119-131.
18. Endres JR., *et al.* "Safety assessment of a proprietary preparation of a novel Probiotic, *Bacillus coagulans*, as a food ingredient". *Food and Chemical Toxicology* 47.6 (2009): 1231-1238.

19. Pearce PJ. "Laboratory Evaluation of Endoscope Water Bottles". *Endo Nurse* (2005).
20. Shida O., et al. "Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov". *International Journal of Systematic Bacteriology* 46.5 (1996): 939-946.
21. Gilmore MS, et al. "The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance". Washington: ASM Press (2002).
22. Fisher K., et al. "The ecology, epidemiology and virulence of *Enterococcus*". *Microbiology* 155.6 (2009): 1749-1757.
23. Ryan KJ., et al. "Sherris Medical Microbiology: An introduction to Infectious Diseases, 4<sup>th</sup> edition". McGraw Hill (2004).
24. Schleifer KH., et al. "Identification of "*Micrococcus candidus*" ATCC 14852 as a strain of *Staphylococcus epidermidis* and of "*Micrococcus caseolyticus*" ATCC 13548 and *Micrococcus varians* ATCC 29750 as Members of a New Species, *Staphylococcus caseolyticus*". *International Journal of Systematic Bacteriology* 32.1 (1982): 15-20.
25. Ogier JC., et al. "Identification of the Bacterial Microflora in Dairy Products by Temporal Temperature Gradient Gel Electrophoresis". *Applied and Environmental Microbiology* 68.8 (2002): 3691-3701.
26. Centers for Disease Control and Prevention (CDC). "Antibiotic Prescribing and Use in Doctor's Offices". Division of Health Care and Quality Promotion (2011).
27. National Committee for Clinical Laboratory Standards. "Performance standards for antimicrobial disk susceptibility tests". Wayne, Penn 22.6 (1997): 40-48.
28. Grabow WOK., et al. "Drug resistant coliforms call for review of water quality standards". *Water Research* 8.1 (1974): 1-9.
29. Levy SB., et al. "Antibacterial resistance worldwide: causes, challenges and responses". *Nature Medicine* 10.12 (2004): S122-S129.
30. World Health Organization. "Overcoming Antimicrobial resistance". *World Health Organization* (2000).
31. Wittwer M., et al. "Genetic diversity and antibiotic resistance patterns in a *Campylobacter* population isolated from poultry farms in Switzerland". *Applied and Environmental Microbiology* 71.6 (2005): 2840-2847.
32. Tran AX., et al. "Resistance to the antimicrobial peptide polymyxin requires myristoylation of *Escherichia coli* and *Salmonella typhimurium* lipid A". *Journal of Biological Chemistry* 280.31 (2005): 28186-28194.
33. Rajpakshe AD., et al. "In vitro Sensitivity of Three Bacterial Pathogens of Koi Carp (*Cyprinus carpio* L.) to Certain Antibiotics". *Journal of Agricultural Science and Technology* 5.2 (2012): 93-98.
34. Kawahara S. "[Chemotherapeutic agents under study]". *Nihon Rinsho* 56.12 (1998): 3096-3099.
35. Pommier Y., et al. "DNA topoisomerases and their poisoning by anticancer and antibacterial drugs". *Chemistry and Biology* 17.5 (2010): 421-433.
36. Girish N., et al. "Extended Spectrum  $\beta$  Lactamase Producing *Klebsiella pneumoniae* and *Escherichia coli* in Neonatal Intensive Care Unit". *Journal of Bacteriology and Parasitology* 3.4 (2012): 141.
37. Odeyemi OA., et al. "In vitro antimicrobial activity of *Aeromonas* spp isolated from estuary using different screening protocols". *International Journal of Pharmaceutical Sciences and Research* 3.2 (2012): 428-433.
38. Jones EM., et al. "Risk factors for antimicrobial resistance in *Escherichia coli* found in GB turkey flocks". *The Veterinary Record* 173.17 (2013): 422.

39. Mølbak K., *et al.* "An outbreak of multidrug-resistant, quinolone-resistant *Salmonella enterica* serotype typhimurium DT104". *The New England Journal of Medicine* 341.19 (1999): 1420-1425.
40. Hawiger J., *et al.* "Antibiotic Sensitivity of *Micrococcus radiodurans*". *Applied Microbiology* 15.2 (1967): 304-306.
41. Voolaid V., *et al.* "Co-occurrence of resistance to different antibiotics among aquatic bacteria". *BMC Microbiology* 8.12 (2012): 225-233.
42. Carballo M., *et al.* "Occurrence of tetracycline residues and antimicrobial resistance in gram negative bacteria isolates from cattle farms in Spain". *Advances in Bioscience and Biotechnology* 4.2 (2013): 295-303.
43. Moulds RF., *et al.* "Gentamicin: a great way to start". *Australian Prescriber* 33 (2010): 35-37.
44. Goljan EF. "Rapid Review Pathology 3<sup>rd</sup> edition". Philadelphia, PA: Elsevier (2011).
45. Dienstag J., *et al.* "In Vitro Studies of Tobramycin, an Aminoglycoside Antibiotic". *Antimicrobial Agents and Chemotherapy* 1.1 (1972): 41-45.
46. Lõivukene K., *et al.* "Prevalence and antibiotic susceptibility of *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* in Estonian intensive care units in comparison with European data". *Scandinavian Journal of Infectious Diseases* 38.11-12 (2006): 1001-1008.
47. Menconi A., *et al.* "Physiological Properties and *Salmonella* Growth Inhibition of Probiotic *Bacillus* Strains Isolated from Environmental and Poultry Sources". *International Journal of Bacteriology* (2013): 8.
48. Katzung BG. "PHARMACOLOGY, 9e. Chapter 44: Chloramphenicol, Tetracyclines, Macrolides, Clindamycin, and Streptogramins". New York: Lange Medical Books (2013).
49. Pechère JC. "Patients' interviews and misuse of antibiotics". *Clinical Infectious Diseases* 15.33 (2001): S170-S173.
50. Shah SQ., *et al.* "Prevalence of Antibiotic Resistance Genes in the Bacterial Flora of Integrated Fish Farming Environments of Pakistan and Tanzania". *Environmental Science and Technology* 46.16 (2012): 8672-8679.
51. Sharma SK., *et al.* "Comparative Study between Penicillin and Ampicillin". *Scholars Journal of Applied Medical Sciences* 1.4 (2013): 291-294.
52. Katzung BG. "Basic and Clinical Pharmacology, 10<sup>th</sup> edition". New York: Lange Medical Books (2007).
53. Greenwood D. "Antimicrobial drugs: chronicle of a twentieth century medical triumph". United Kingdom: Oxford University Press (2008).
54. British National Formulary (57<sup>th</sup> Edition). Pharmaceutical Press (2009).
55. Phillips I., *et al.* "Importance of beta-lactamase induction". *European Journal of Clinical Microbiology and Infectious Diseases* 12.1 (1993): S19-S26.
56. McEvoy GK. "AHFS Drug Information 95". Bethesda, MD: American Society of Hospital Pharmacists, Inc (1995).
57. Sweetman SC. "Martindale: The Complete Drug Reference, 36<sup>th</sup> edition.". London: Pharmaceutical Press (2009).
58. Li D., *et al.* "Antibiotic resistance characteristics of environmental bacteria from an oxytetracycline production wastewater treatment plant and the receiving river". *Applied and Environmental Microbiology* 76.11 (2010): 3444-3451.

59. Mandal S., *et al.* "R-factor in Salmonella enterica serovar Typhi: Transfer to and acquisition from Escherichia coli". *Japanese Journal of Infectious Diseases* 56 (2003): 65-67.
60. Mandal S., *et al.* "Plasmid-encoded multidrug resistance of Salmonella typhi and some enteric bacteria in and around Kolkata, India: a preliminary study". *Online Journal of Health and Allied Sciences* 3.4 (2004): 2-7.

**Volume 16 Issue 6 June 2020**

**© All rights reserved by Dareen El Shareef Ahmed., *et al.***