PREVALANCE OF ANTIBIOTIC RESISTANCE IN MARINE BACTERIA FROM THE COASTAL ENVIRONMENT

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DATE: APRIL 2023

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DECLARATION

I hereby declare that the data presented in this Dissertation report entitled, **"PREVALANCE OF ANTIBIOTIC RESISTANCE IN MARINE BACTERIA FROM THE COASTAL ENVIRONMENT"** is based on the results of investigations carried out by me in the Marine Microbiology at the School of Earth, Ocean and Atmospheric Sciences, Goa University under the Supervision of Dr. Priya M. D'Costa and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report **"PREVALANCE OF ANTIBIOTIC RESISTANCE IN MARINE BACTERIA FROM THE COASTAL ENVIRONMENT"** is a bonafide work carried out by Miss. Nidhi Digambar Kambli under my supervision in partial fulfilment of the requirements for the award of the degree of Master's Degree in the Discipline Marine Microbiology at the School of Earth, Ocean and Atmospheric Sciences, Goa University.

Dr. Priya M. D'Costa

Marine Microbiology

Date: APRIL 2023

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ACKNOWLEGDEMENT

It gives me immense pleasure to present my dissertation entitled “Prevalence of antibiotic resistance in marine bacteria from the coastal environment.”

Firstly, I would like to express my deep sense of gratitude and would like to thank my guide, Dr. Priya M. D’Costa, Marine Microbiology, School of Earth, Ocean and Atmospheric Sciences, for her valuable guidance, support, and untiring effort during the course of the dissertation. She has been a great source of motivation and encouragement for me to take up this topic as my study.

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Nidhi Kambli

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Antibiotics are antimicrobial agents that work against microorganisms, mainly bacteria. The most prevalent type of antibacterial agent used to treat and prevent bacterial infections is antibiotics. They can either kill or suppress bacterial growth. Only a few antibiotics have antiprotozoal action. Antibiotics are ineffective against viruses such as the common cold or influenza; therefore, medications that inhibit viral growth are referred to as antiviral drugs or antivirals rather than antibiotics. Many antibiotics are ineffective against fungi; antifungal drugs are those that inhibit the development of fungi (Gould, 2016).

Antibiotics over the Years

The first known use of antibiotic substances was by the ancient Chinese over 2,500 years ago. Curative properties of moldy soyabeans were discovered by the Chinese and these substances were used to treat pimples, carbuncles and similar infections. Several ancient civilizations treated infections using these molds. Now, it is known that molds and plants produce bioactive metabolites or antibiotics. However, at the time, no compounds were known to exert antibiotic effects (Maurois, 1959).

In 1665, Robert Hooke first did the microbial observation. He became the first scientist to use a simple microscope to observe microbes and fungi, in addition to small organisms and plant structures.
Looking at a thin cut of cork, Hook claims that the smallest building elements of life are microscopic "kits" known as cells. Hooke's finding ushered in the cellular theory, which holds that all living things are made up of cells (Kourkouta et al. 2018). A few years later, in 1674, after he built a microscope with his lens zooming from X300 to X500, Anton von Leeuwenhoek started studying protozoa and other microbes. With some of the 400 microscopes he created, he was the first to successfully observe living microbes (Fasoulakis, 2016). Based on his studies before the Royal Society of London in 1676,

he stated that the microorganisms he examined with a big lens were alive. Van Leeuwenhoek drew precise designs for these aquatic creatures and on his facial skin and teeth. The first bacteria and protozoa were depicted in these designs. But researchers at the time were forbidden to use microscopes, so microbiology really took off 200 years later when Louis Pasteur and Robert Koch succeeded in linking microbes to infectious diseases. Pasteur established the fundamental principles of microbiology in 1859. In 1876, Koch demonstrated that anthrax was associated with anthrax and ultimately in 1882 he linked tuberculosis to tuberculosis mycobacteria, which were later discovered to be linked (Partalidou, 2016).

Pseudomonas aeruginosa cultivation yielded the first antibiotic, pyocyanase which was discovered by Rudolph Emmerich and Oscar Löw, two German academics in the late 1890s and was used to treat patients suffering from cholera and typhus with dubious effectiveness. Paul Ehrlich developed the arsenic-based medication Salvarsan in 1909, which worked against *Treponema pallidum*, the microorganism responsible for syphilis. This discovery set the groundwork for future antimicrobial agent development. However, Alexander Flemming's 1928 discovery of penicillin, which is still used in clinical therapies today, was a watershed moment in the creation of antimicrobial drugs. In France in 1897, Ernest Duchesne identified the antibacterial activities of *Penicillium sp*. However, until Alexander Fleming found penicillin, his findings had little impact on the scientific world. In 1932, Bayer's research team discovered the first sulphonamide, prothosyl, and Gerhard Domagk developed its efficiency against severe bacterial illnesses the following year. Penicillin, a product of the *Penicillium* fungus, was the first antibiotic accessible to doctors in 1946. It is referred to as a "child of war" because substantial research and observations were undertaken previous to its development during WWII. The discovery of penicillin was regarded as a miracle of modern times because it was able to cure all types of illnesses produced by staphylococci and streptococci. These two bacteria are

responsible for the largest number of infections known; it is understandable to feel relieved by this discovery. The utilization of streptomycin and tetracycline was found in 1940s and early 1950s, and antibiotic chemotherapy is widely acknowledged in the field of clinical medicine. These antibiotics are efficient against a wide range of bacteria that cause disease, including tuberculosis bacteria. Scientists initially became interested in the existence of drugs, particularly hormones, in the environment in the course of the 1970s. The study of environmental pollutants such as heavy metals, pesticides, aromatic polycyclic hydrocarbons, chlorinated dioxins, and detergents received a lot of attention in the 1980s despite a decline in interest in hormones. Other compounds like analgesics, anti-rheumatic medicines, and antibiotics have also been included to the research list since the mid1990s (Nikiforou & Kinki 2013).

The introduction of antibiotics and their clinical use in medical breakthroughs were of greatest success (Katz et al. 2016). In addition to treating infections, antibiotics have enabled many modern medical procedures, such as cancer treatment, organ transplantation, and open-heart surgery. However, abuse of valuable compounds has led to a rapid rise in Antimicrobial Resistance (AMR), making some infections virtually untreatable (Prescott 2014). Antimicrobial Resistance (AMR) is the potential of microorganisms to defend themselves against the effects of medicines to which they used to be vulnerable. Bacteria become resistant to antibiotics through genetic mutation or acquisition of Antibiotic Resistance Genes (ARGs). In nature, bacteria are thought to produce antibiotics that kill or inhibit neighboring bacteria as a strategy to conserve resources (D'Costa et al. 2007). When resources such as nutrients are limited, bacteria produce antibiotics to destroy or inhibit neighboring bacteria, reducing competition for scarce resources. For this strategy to be effective, antibiotic-producing bacteria must be able to survive by possessing mechanisms of resistance to the antibiotics they produce. Thus, antibiotic resistance continues to pose a growing threat to global public health by

distorting the treatment of infections caused by virtually every major pathogen that can be transferred to (Séveno et al. 2002).

The perils of the post-antibiotic era led policymakers to recognize this threat to human health and pledge additional funding, leading to a gradual resurgence of interest in antibiotic discovery and development (Walsh et al. 2014).

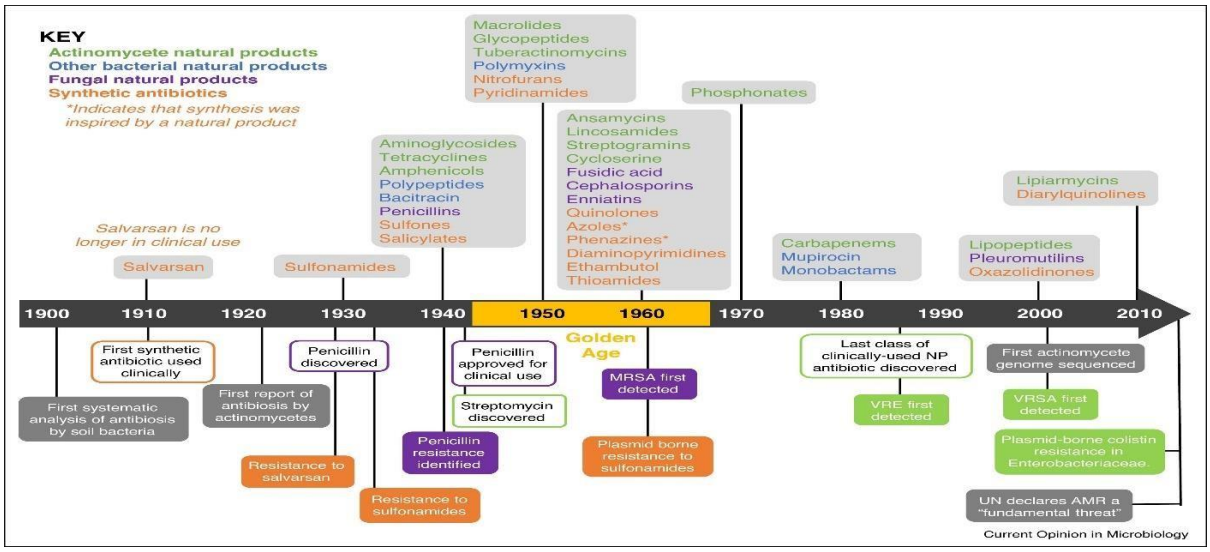


Fig1.1: Timeline showing the decade new classes of antibiotic reached the clinic (Hutchings et al. 2019).

Consequences of the widespread uses of antibiotics

Antimicrobial agents are widely used in medicine and agriculture to treat various medical conditions. However, concerns have been raised about their indiscriminate or poorly regulated use and the negative impact this may have on the environment. A major consequence of antibiotic use and misuse is the emergence and spread of antibiotic resistance. As it affects the welfare of animals and food safety, this is a public health issue with obvious implications for both human and veterinary medicine.

Concern over the emergence of antibiotic resistance is developing on a global scale, especially in case of antibiotics considered first-line treatments for certain human infections, prompting measures to monitor bacterial antimicrobial resistance in humans, animals, and bacteria (Smaldone et al. 2014).

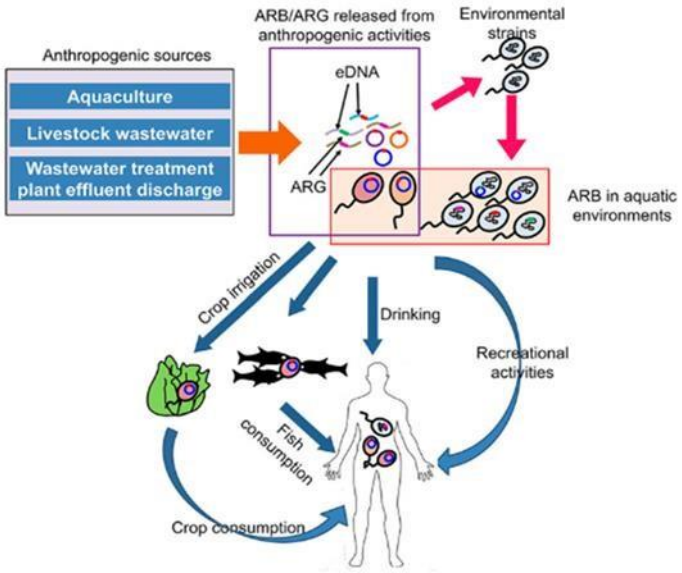


Fig1.2: Human health risks caused by antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) in aquatic environments (Amarasiri et al. 2020).

Given the clinical importance of this issue, it is not surprising that for many years, research on antibiotic-resistant bacteria and resistance mechanisms was almost entirely concentrated on pathogens in clinical settings. However, it has recently been discovered that antibiotic resistance is prevalent not only among commensal bacteria in humans and animals, but also among environmental bacteria. The latter finding is significant because bacteria in natural environments are likely to function as a reservoir of resistance genes that can be passed on to pathogenic species. Therefore, this information may provide early warning of future clinically relevant resistance mechanisms and facilitate the development of effective new drugs (Riesenfeld et al. 2004).

A number of terrestrial and aquatic environments have been examined for the presence of antibioticresistant bacteria, and the mechanisms of resistance in these bacteria have been described in

some cases. However, little is known about antibiotic resistance in Earth's deep underground environment, which has only been found to contain bacteria in recent decades. These environments were considered devoid of microbial life until the mid-1980s, when several research groups found significant numbers of bacteria in shallow aquifers less than 30 meters below the earth's surface. Since then, bacteria have been shown to live in a variety of environments chemically and physically, at depths of at least 3.6 km (Fredrickson et al. 2006; Brown et al. 2009).

There is also concern that the diversity and prevalence of antimicrobial resistance in the environment were underestimated and could have widespread impacts. The aquatic environment is considered an ideal environment for the acquisition and spread of antimicrobial resistance, and human exposure to antimicrobial-resistant bacteria (ARBs) and antimicrobial resistance genes (ARGs) in the aquatic environment It has a high and diverse bacterial load and is a known sink for a wide variety of clinical bacteria and contaminants. Water bodies are considered one of the reservoirs and transmission routes for the spread of antibiotic-resistant bacteria (Amarasiri et al. 2020). Marine ecosystems, in particular, are not only important reservoirs of AMR, but also facilitate their formation (Al-Sarawi et al.2018). Quantitative microbial risk assessment (QMRA) has been proposed as an appropriate method to assess and quantify this health risk. However, information on exposure to ARBs and ARGs in the aquatic environment is lacking in many scenarios, and dose-response models for ARB infection have not yet been developed (Amarasiri et al. 2018).

AIMS AND OBJECTIVES

1.2 AIMS & OBJECTIVES

Antibiotic-resistant microorganisms as well as novel resistance mechanisms have been screened in a number of environmental habitats (Brown & Balkwill 2009). Abuse of valuable compounds has led to a rapid rise in Antimicrobial Resistance (AMR), making some infections virtually untreatable

(Prescott 2014). Antibiotic Resistance in coastal bacterial isolates is significant for various reasons. Antibiotic resistance is an increasing problem around the world, and studying the antibiotic susceptibility of coastal bacterial isolates can also provide valuable information about the prevalence of susceptibility in coastal habitats. This data can be used to create strategies to combat the increase in antibiotic resistance. Thus, the aim of the present study is to investigate the prevalence of antibiotic resistance in marine bacteria from the coastal environment.

The objectives are:

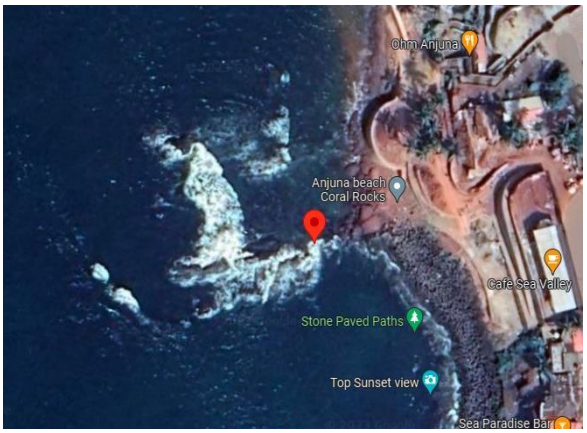
1. To isolate bacteria from different coastal environments.
2. To investigate sensitivity of marine bacterial isolates to different antibiotics.

CHAPTER 2

MATERIALS AND METHODOLOGY

2.1 SAMPLE COLLECTION AND ISOLATION OF BACTERIA FROM COASTAL ENVIRONMENT

I. SAMPLING



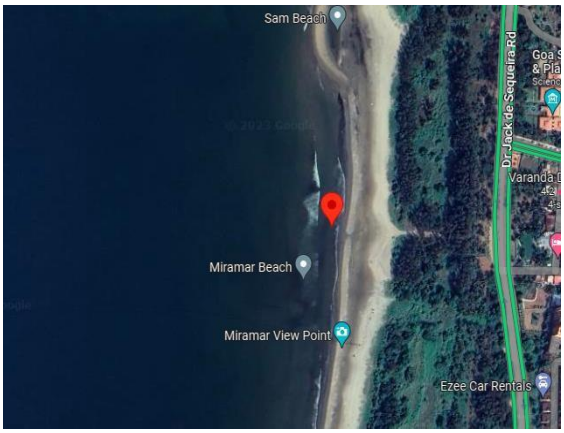


Fig 2.1: A) Sampling station at Anjuna Beach **Fig 2.2 B) Sampling Station at Miramar Beach**

This study consists of two sampling stations from the coastal environment i.e.,1) Anjuna Beach (15°35'03.6"N 73°44'12.7"E), sampled on 11th January 2023 and 2) Miramar Beach (15°28'34.3"N 73°48'22.5"E) sampled on 16th January 2023. Seawater samples were collected in sterile 50ml Tarsons tubes. Sediment sample were also collected using a sterile spatula in sterile 50ml Tarsons tubes. The samples were kept in polystyrene box containing ice till they were brought to the laboratory for further processing.

II. PROCESSING OF SAMPLE FOR VIABLE COUNT AND ISOLATION OF BACTERIAL CULTURES

Fig 2.3: Isolation of microorganisms from seawater

After transporting the sample to the laboratory, the Tarsons tubes containing samples were surface sterilized using sanitizer and opened under sterile condition in the laminar air flow (both seawater and sediment samples), for analysis of Viable Count of bacteria.

2.2 VIABLE COUNT OF BACTERIA

The viability of a microbial population is the ratio of its viable count to the total concentration of microbes, dead or alive. A viable organism is one that is capable of multiplying to produce two or more offspring under optimal conditions for the species and strain of microbe concerned (Postgate 1969).

PRINCIPLE

A viable plate count, also known as a plate count, is a measure of viable or living cells. It is founded on the principle that when viable cells are incubated in suitable conditions, they replicate and form visible colonies. On a plate, a measured quantity of liquid culture is inoculated. After incubating the plate, the colonies that form are enumerated. Because multiple cells may have landed on the same spot to create a single colony, the results are typically expressed as colony-forming units per millilitre (CFU/mL) rather than cells per millilitre (CFU/mL). Furthermore, bacteria grown in clusters or chains are difficult to disperse, and a single colony may reflect several original cells. Some cells are viable

but nonculturable, meaning they will not create colonies on solid media. Because of these factors, the viable plate count is regarded as a low estimate of the actual number of live cells. These constraints do not diminish the method's utility in providing estimates of live bacterial numbers.

There are two techniques for inoculating plates for viable counts: the pour plate method and the spread plate method. Although the final inoculation process varies, both begin with a serial dilution of the culture. Because the concentration of cells in even a slightly turbid culture is too high to create discrete colonies that can be counted on a plate, serial dilution is required. A culture's serial dilution is an essential first step before using the pour plate or spread plate method. The objective of the serial dilution process is to acquire plates with CFUs in the range of 30-300, and the process typically consists of several dilutions in multiples of 10 to facilitate calculation. A preliminary approximation of the culture density is used to determine the number of serial dilutions (Kay et al. 2018).

Viable Count Procedure:

The seawater samples were serially diluted in sterile saline solution from 10^{0} to 10^{-3} and further 0.1ml was spread plated on sterile Zobell Marine Agar (ZMA) and sterile Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS). Similarly, the sediment samples were suspended in 1ml of sterile saline (0.1 $gmml^{-1}$), mixed and serially diluted up to 10^{-3} and 0.1ml was spread plated on sterile ZMA and TCBS Agar plates. The ZMA plates were incubated for 24 hours and the TCBS plates were incubated for up to 48 hours at room temperature. The number of colonies were counted to calculate the total viable count. The results were depicted graphically.

Maintenance of bacterial cultures

Morphologically different colonies were selected from the ZMA and TCBS plates mentioned above and purified in three cycles. Colony characteristics were noted down. Sterile Nutrient agar slants were prepared, cultures were streaked on them, allowed to grow at room temperature for 24/48 hours, wrapped with parafilm and stored in the fridge at 4ºC until further use.

2.3 EFFECT OF ANTIBIOTICS ON BACTERIA

Experimental plan:

The 20 isolates were tested for their resistance to 2 different antibiotics i.e., 1) ROSCILLIN 500MG INJECTION (ampicillin) and 2) AZEE 500MG INJECTION (Azithromycin).

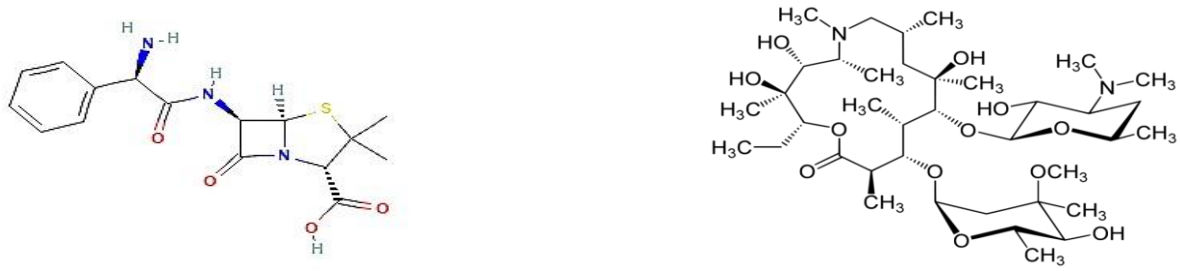
ROSCILLIN contains ampicillin, which belongs to the antibiotic group of medicines. It is used to treat bacterial infections of ear, nose, throat, respiratory tract (bronchi, lungs), urinary tract, genitals, stomach, blood, brain, skin or soft tissue. It can also be used to treat typhoid fever, meningitis (inflammation of the lining of the brain), peritonitis (inflammation of the lining of the stomach), and endocarditis (inflammation of the lining of the heart and heart valves).

Mode of action: It is a bactericidal agent. It kills the susceptible bacteria by blocking the bacterial cell wall synthesis that is essential for the bacterial cell to survive. This action destroys the susceptible bacteria, prevents their further growth and multiplication, and reduces the severity of the infection.

AZEE is an antibiotic called as azithromycin which is used to treat various types of bacterial infections such as infections of the respiratory tract, ear, nose, throat, skin, and eye in adults and children. It may also be effective in some sexually transmitted diseases like gonorrhea. It is also used to treat infections of the pelvic area and reproductive tract in women. It is a broad-spectrum type of

antibiotic effective in killing many types of gram-positive bacteria, some types of gram-negative bacteria, and other microorganisms.

Mode of Action: It treats bacterial infections by disrupting the bacteria's protein synthesis process and stopping their growth. It stops the process by binding to a particular part of the nucleus of the bacterial cell and preventing the growth process. Therefore, based on the concentration of Azithromycin & organism, the bacteria are either killed or their growth is slowed down.



(A) **(B)**

Fig 2.4: chemical structure of Ampicillin and Azithromycin

The microbial susceptibility is tested to determine the potential effectiveness of specific antibiotic on the bacteria or to determine if the bacteria is resistant to certain antibiotics.

Procedure:

Under sterile conditions in the Biosafety cabinet, a loop full of pure culture was inoculated in test tubes containing 5ml of sterile nutrient broth. The tubes are incubated at room temperature for 24 hours for growth. This was done for all 20 isolates. The freshly grown 24 hours old cultures were used to check their resistance against the two antibiotics i.e., Ampicillin (50 µg/ml) and Azithromycin (50 µg/ml).

Two set of tubes were taken, labelled with their respective culture codes for all 20 isolates as experimental and positive control. Two replicates were maintained. The positive control tubes contained 5ml sterile nutrient broth with 0.1ml 24-hour old culture inoculated in it, whereas the experimental tubes contained 5ml of sterile nutrient broth with antibiotic and 0.1ml of 24hours old culture inoculated in it. Also, a tube of negative control was maintained containing 5ml sterile nutrient broth with antibiotic with no culture. All the tubes were incubated at room temperature for 24hours, followed by monitoring of growth calorimetrically. Before starting the experiment, the OD of 24 hours old cultures was checked to ensure the growth of bacterium. The optical density (OD) was measured at 620nm using a BR Biochem Life Sciences Pvt. Ltd calorimeter. The calorimeter is calibrated to autozero using the negative control and O.D is measured for all the positive and experimental test-tubes to check the susceptibility of bacteria to the antibiotics.

2.4 CHARACTERIZATION OF BACTERIAL CULTURES

For these tests, 24-hour old bacterial cultures were used.

GRAM STAINING

Principle:

Grams Stain is used for differentiation of bacteria on the basis of their gram nature (Tripathi & Sapra, 2020). It is the most widely used differential staining method in all microbiology laboratories. This is one of the most important criteria in any kind of bacterial isolate identification scheme. Various mechanisms have been proposed to explain the Gram reaction. There are many physiological differences between gram-positive and gram-negative cell walls. Since Christian Gram's discovery of Gram staining, this process has been extensively studied and redefined. In practice, thin smears of bacterial cells are stained with crystal violet and treated with an iodized mordant to increase the binding of the primary stain. Use a destaining solution of alcohol or acetone to remove crystal violet from cells that are weakly associated with it. Next, use a counterstain (such as safranin) to create a color contrast in the destained cells. Gram-positive bacteria have a thick reticulated cell wall of peptidoglycan (50–90% of the cell envelope) that stains purple with crystal violet, whereas Gramnegative bacteria have a thinner layer (10% of the cell envelope), which does not retain the purple stain and is counterstained pink with safranin. In smears properly stained by the Gram staining procedure, Gram-positive bacteria appear blue to purple, while Gram-negative cells appear pink to red.

Procedure:

On a clear dry glass slide, a smear of a 24-hour-old bacterial culture was prepared. The slide was allowed to air dry and gently heat fixed. The slide was flooded with Gram’s crystal violet for 1 minute.

The smear was then flooded with Gram's Iodine and allowed to remain for 1 minute. Iodine was decolourized with Gram's Decolourizer until the purple dye no longer flowed from the smear. It was then washed with tap water. The smear was counter stained using safranin for 20 seconds and rinsed off with water. The slide was air dried and examined under oil immersion objective.

2.5 CATALASE TEST

Organisms must depend on defense mechanisms to repair or avoid the oxidative damage caused by hydrogen peroxide (H2O2) in order for them to survive. Some bacteria generate catalase, an enzyme that aids in cellular detoxification. Catalase counteracts hydrogen peroxide's bactericidal effects, and its concentration in microbes has been linked to pathogenicity.

The catalase test plays a role in determining whether bacteria are producing the enzyme catalase. It is important to distinguish between catalase-negative *Streptococcaceae* from catalase-positive *Micrococcaceae*. Although it is most beneficial in distinguishing between genera, it is also helpful in identifying certain gram-positive organisms such as *Aerococcus viridians* (negative) from *Aerococcus urinae* (positive) and gram-negative organisms from other *Campylobacter* species, such as *Campylobacter foetus*, *Campylobacter jejuni*, and *Campylobacter coli* (all positive). Its utility in the presumptive differentiation of certain *Enterobacteriaceae* has been documented by some. The catalase test is also useful in distinguishing between aerobic and obligate anaerobic microbes, as anaerobes are known to lack the enzyme. In this situation, the catalase test is useful for distinguishing aerotolerant *Clostridium* strains that are catalase negative from *Bacillus* strains that are catalase positive (Reiner 2010).

Nutrient Agar plate method:

Sterile nutrient agar plates were prepared and inoculated with desired cultures. The plates were incubated at room temperature for 24 hours. On the 24 hours old heavily inoculated pure cultures 0.1ml of H2O2 (Hydrogen peroxide) was added on the nutrient agar plates. The plates were placed against a dark background and observed for immediate bubble formation. Positive reactions are evident by immediate effervescence (bubble Formation). No bubble formation (no catalase enzyme to hydrolyze the hydrogen peroxide) represents a catalase-negative reaction.

2.6 MOTILITY

Motility test is a test done to determine the motility of microorganism by the soft agar butt stab method. The sterile nutrient soft agar butt are prepared and cultures were stab inoculated. All the tubes were incubated at room temperature for 24 hours. Tubes were checked to see if cultures are motile (indicated by diffused growth in butt) or non-motile (non-diffused growth).

CHAPTER 3

RESULTS AND DISCUSSION

3.1 ISOLATION OF CULTURES

Twenty morphologically distinct colonies were isolated; 9 isolates were from Zobell Marine Agar and 11 isolates were from TCBS Agar.

Table 1: Isolated cultures on ZMA and TCBS

ISOLATED ON			
ZMA		TCBS	
SR.NO	CULTURE CODE	SR.NO	CULTURE CODE
1	M1	1	TM3
2	M2	2	TM4
3	M3	3	TM5
4	M4	4	TM6
5	M5	5	TM7
6	M6	6	TM8
7	M8	7	TM9
8	M9	8	TM10
9	V1	9	TV3
		10	TV5
		11	TV8

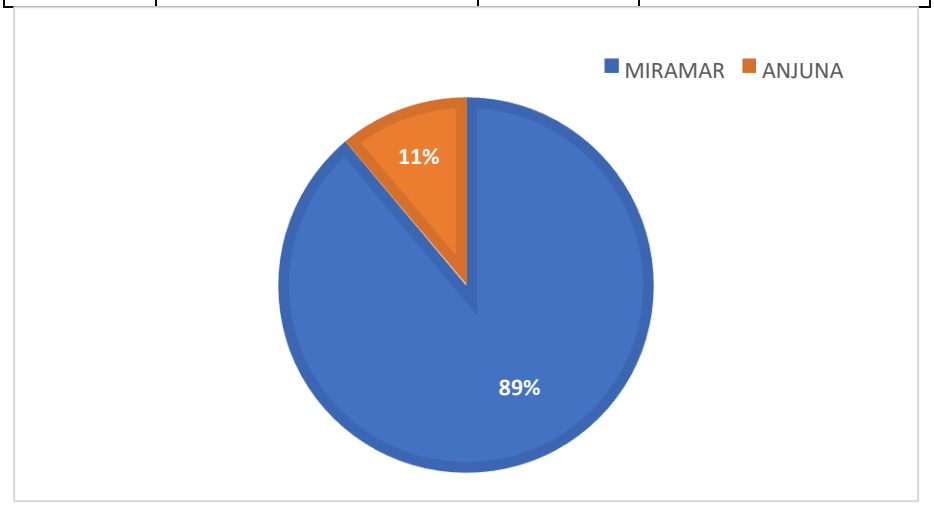


Fig 3.1: Source of isolation of heterotrophic bacterial isolates on ZMA.

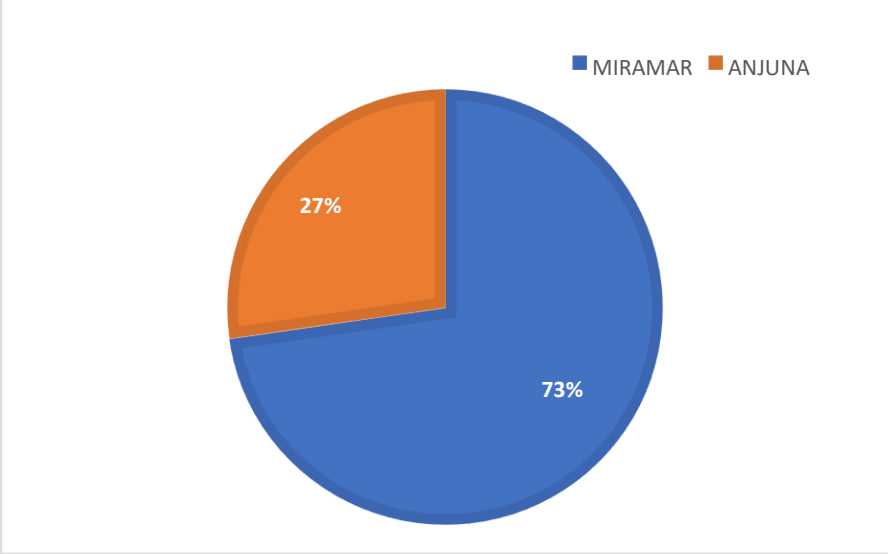


Fig 3.2: source of isolation of *Vibrio* spp. on TCBS.

Majority of the heterotrophic bacterial cultures and *Vibrio* spp. were isolated from Miramar (**Fig 3.1 & 3.2**).

3.2 VIABLE COUNT

The viable count could not be calculated since the number of colonies did not fall in the range of 30-300 CFU/ml.

3.3 COLONY CHARACTERISTICS OF ISOLATES

Table 2: Colony characteristics of isolates.

CULTURE	M1	M2	M3
Medium	ZMA	ZMA	ZMA
Time	24hrs	24hrs	24hrs
Temperature	Room Temperature	Room Temperature	Room Temperature
Shape	Circular	Circular	Circular
Size	Pinpoint	Pinpoint	Small
Colour	White	Yellow	White
Margin	Entire	Entire	Entire
Elevation	Convex	Convex	Raised
Surface Texture	Smooth	Smooth	Smooth

Consistency	Butyrous	Butyrous	Butyrous
Opacity	Opaque	Opaque	Opaque
Gram Character	Gram-Negative Cocci	Gram-Negative Cocci	Gram positive cocci
Motility	Motile	Non-Motile	Non-Motile

Table 2 (continued): .

Colony characteristics of isolates

CULTURE	M4	M5	M6
Medium	ZMA	ZMA	ZMA
Time	24hrs	24hrs	24hrs
Temperature	Room Temperature	Room Temperature	Room Temperature
Shape	Circular	Circular	Circular
Size	Small	Small	Pinpoint
Colour	Yellow	White	Yellow
Margin	Entire	Entire	Entire
Elevation	Raised	Raised	Raised
Surface Texture	Smooth	Smooth	Smooth
Consistency	Slimy	Butyrous	Butyrous
Opacity	Opaque	Opaque	Opaque
Gram Character	Gram-Negative Cocci	Gram-Negative Cocci	Gram-Negative Coccobacilli
Motility	Motile	Motile	Motile

Table 2 (continued): .

Colony characteristics of isolates

CULTURE	M8	M9	V1
Medium	ZMA	ZMA	ZMA
Time	24hrs	24hrs	24hrs
Temperature	Room Temperature	Room Temperature	Room Temperature
Shape	Circular	Circular	Circular
Size	Small	Small	Small
Colour	Creamy	Creamy	Yellow
Margin	Entire	Entire	Entire
Elevation	Raised	Raised	Raised
Surface Texture	Smooth	Smooth	Smooth
Consistency	Butyrous	Butyrous	Butyrous
Opacity	Opaque	Opaque	Opaque
Gram Character	Gram-Positive Cocci	Gram-Positive Cocci	Gram-Negative Cocci
Motility	Non-Motile	Non-Motile	Non-Motile

Colony characteristics of isolates

CULTURE	TM3	TM4	TM5
Medium	TCBS	TCBS	TCBS
Time	48hrs	48hrs	48hrs

Table 2 (continued): .

Temperature	Room Temperature	Room Temperature	Room Temperature
Shape	Circular	Circular	Circular
Size	Small	Large	Moderate
Colour	Green	Grey Green	Yellow
Margin	Entire	Entire	Entire
Elevation	Raised	Raised	Raised
Surface Texture	Smooth	Smooth	Smooth
Consistency	Slimy	Slimy	Slimy
Opacity	Opaque	Opaque	Opaque
Gram Character	Gram-Positive rods	Gram-Negative coccobacilli	Gram-Negative coccobacilli
Motility	Non-Motile	Motile	Motile

NOTE: Isolate TM4 and TM5 showed green pigment production when grown in Zobell Marine Broth and Nutrient agar.

Colony characteristics of isolates

CULTURE	TM6	TM7	TM8
Medium	TCBS	TCBS	TCBS

Table 2 (continued): .

Time	48hrs	48hrs	48hrs
Temperature	Room Temperature	Room Temperature	Room Temperature
Shape	Circular	Circular	Circular
Size	Small	Pinpoint	Small
Colour	Yellow	Green	Green
Margin	Entire	Entire	Entire
Elevation	Raised	Convex	Raised
Surface Texture	Smooth	Smooth	Smooth
Consistency	Slimy	Slimy	Slimy
Opacity	Opaque	Opaque	Opaque
Gram Character	Gram-Positive short rods	Gram-Negative Coccobacilli	Gram-Negative short rods
Motility	Non-Motile	Non-Motile	Non-Motile

Table 2(continued): Colony characteristics of isolates

CULTURE	TM9	TM10	TV3
Medium	TCBS	TCBS	TCBS
Time	48hrs	48hrs	48hrs
Temperature	Room Temperature	Room Temperature	Room Temperature
Shape	Circular	Circular	Circular
Size	Small	Moderate	Small
Colour	Yellow	Green	Green
Margin	Entire	Entire	Entire
Elevation	Raised	Flat	Raised
Surface Texture	Smooth	Smooth	Smooth
Consistency	Slimy	Slimy	Slimy
Opacity	Opaque	opaque	Opaque
Gram Character	Gram-Positive short rods	Gram-Positive bacilli	Gram-Positive short rods
Motility	Motile	Non-Motile	Motile

Table 2(continued): Colony characteristics of isolates

CULTURE	TV5	TV8
Medium	TCBS	TCBS
Time	48hrs	48hrs
Temperature	Room Temperature	Room Temperature
Shape	Circular	Circular
Size	Small	Small
Colour	Green	Yellow
Margin	Entire	Entire

Elevation	Raised	Flat
Surface Texture	Smooth	Smooth
Consistency	Slimy	Slimy
Opacity	Opaque	Opaque
Gram Character	Gram-Positive Bacilli	Gram-Positive Bacilli
Motility	Motile	Motile

3.4 DETERMINATION OF GRAM CHARACTERISTICS, MORPHOLOGY, MOTILITY AND CATALASE TEST OF THE ISOLATES

SR.NO	CULTURE	GRAM CHARACTER AND MORPHOLOGY	MOTILITY	CATALASE TEST
1)	M1	Gram negative cocci	Motile	Positive
2)	M2	Gram negative cocci	Non-motile	Positive
3)	M3	Gram positive cocci	Non-motile	Positive
4)	M4	Gram negative cocci	Motile	Positive
5)	M5	Gram positive cocci	Motile	Positive
6)	M6	Gram negative coccobacilli	Motile	Positive
7)	M8	Gram positive cocci	Non-motile	Positive
8)	M9	Gram positive cocci	Non-motile	Positive
9)	V1	Gram negative cocci	Non-motile	Positive
10)	TM3	Gram positive short rods	Non-motile	Positive
11)	TM4	Gram negative coccobacilli	Motile	Positive
12)	TM5	Gram negative coccobacilli	Motile	Positive
13)	TM6	Gram positive short rods	Non-motile	Positive
14)	TM7	Gram negative coccobacilli	Non-motile	Positive
15)	TM8	Gram negative short rods	Non-motile	Positive
16)	TM9	Gram positive short rods	Non-motile	Positive
17)	TM10	Gram positive bacilli	Non-motile	Positive
18)	TV3	Gram positive short rods	Motile	Positive

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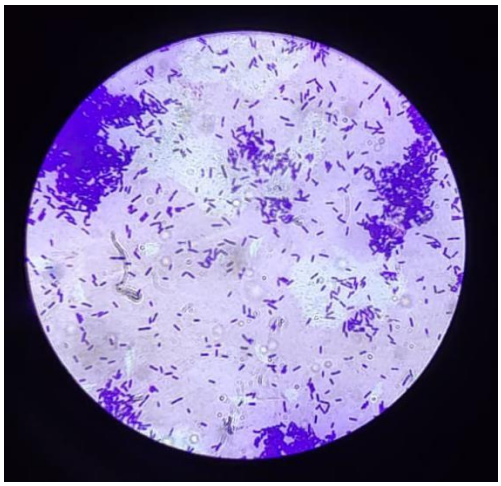
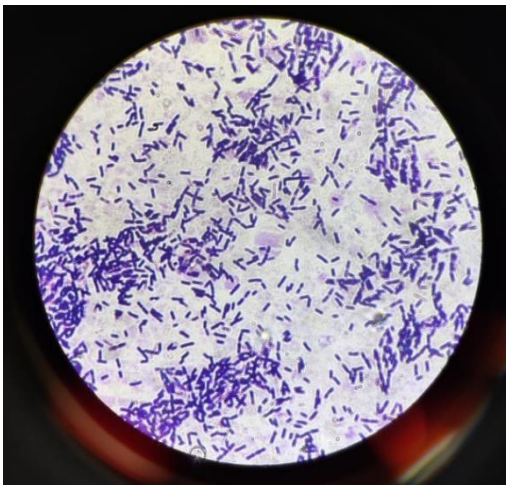
19)	TV5	Gram positive bacilli	Motile	Positive
20)	TV8	Gram positive bacilli	Motile	Positive

Table 3: Determination of gram characteristics, morphology, motility and catalase test of the isolates.

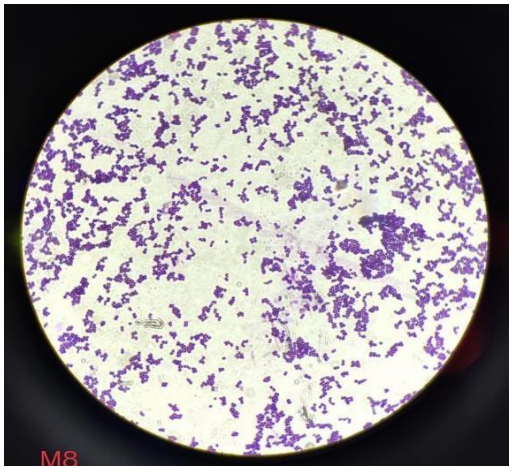
From the 20 heterotrophic bacteria and *Vibrio* spp. isolated M1, M2, M4 and V1 isolates were Gramnegative cocci. Isolates M3, M5, M8 and M9 were Gram-positive cocci. Isolates M6, TM4, TM5 and TM7 were Gram-negative coccobacilli. Isolates TM3, TM6, TM9 and TV3 were Gram positive short rods. TM10, TV5 and TV8 were Gram-positive bacilli and isolate TM8 was Gram-negative short rods. Also isolates M1, M4, M5, M6, TM4, TM5, TV3, TV5, TV8 are motile and isolates M2, M3, M8, M9, V1, TM3, TM6, TM7, TM8, TM9, TM10 were found to be non-motile. All of the 20 isolates showed immediate effervescence/bubble formation indicating presence of catalase activity.



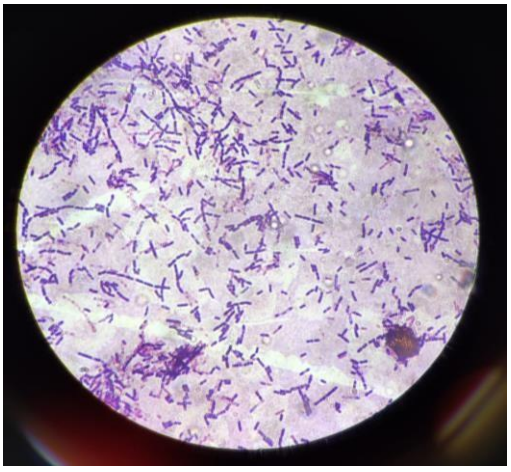
TM3: Gram-positive short rods



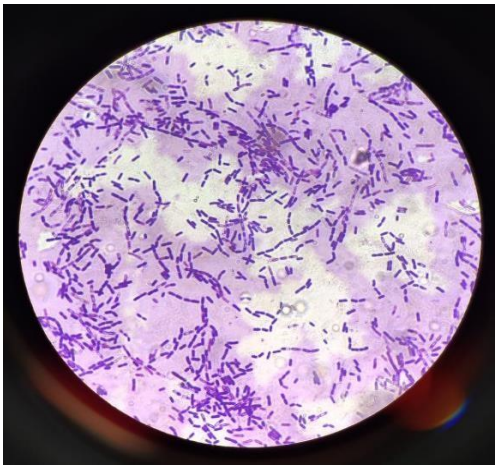
TM6: Gram-positive short rods



M8: Gram-positive cocci



TV5: Gram-positive bacilli



TV3: Gram-positive short rods Fig 3.3: Gram characteristics of selected bacterial isolates

Fig 3.4: Indication of positive catalase test by immediate effervescence

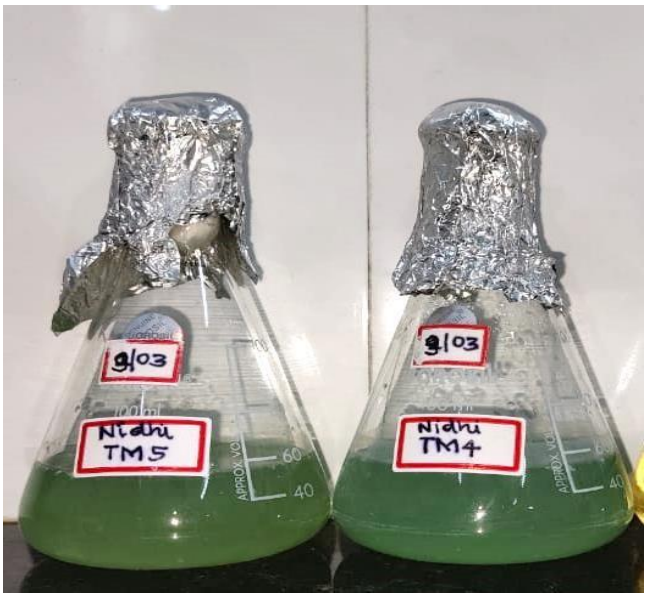


Fig3.5: Bacterial isolates (TM4, TM5) showing pigment production in flask grown in ZMB.

3.5 EFFECTS OF ANTIBIOTICS ON HETEROTROPHIC BACTERIA AND *Vibrio* spp.:

Table 4: OD (at 620nm) of bacterial culture grown in nutrient broth for 24-hours.

SR.NO	CULTURE CODE	OD AT 620nm	SR.NO	CULTURE CODE	OD AT 620nm
1)	M1	0.979	**11)**	TM4	0.688
2)	M2	0.962	**12)**	TM5	0.845
3)	M3	0.893	**13)**	TM6	0.701
4)	M4	0.879	**14)**	TM7	0.752
5)	M5	1.085	**15)**	TM8	0.918
6)	M6	0.793	**16)**	TM9	0.774
7)	M8	1.174	**17)**	TM10	0.826
8)	M9	1.191	**18)**	TV3	0.695
9)	V1	0.944	**19)**	TV5	0.881
10)	TM3	0.797	**20)**	TV8	0.707

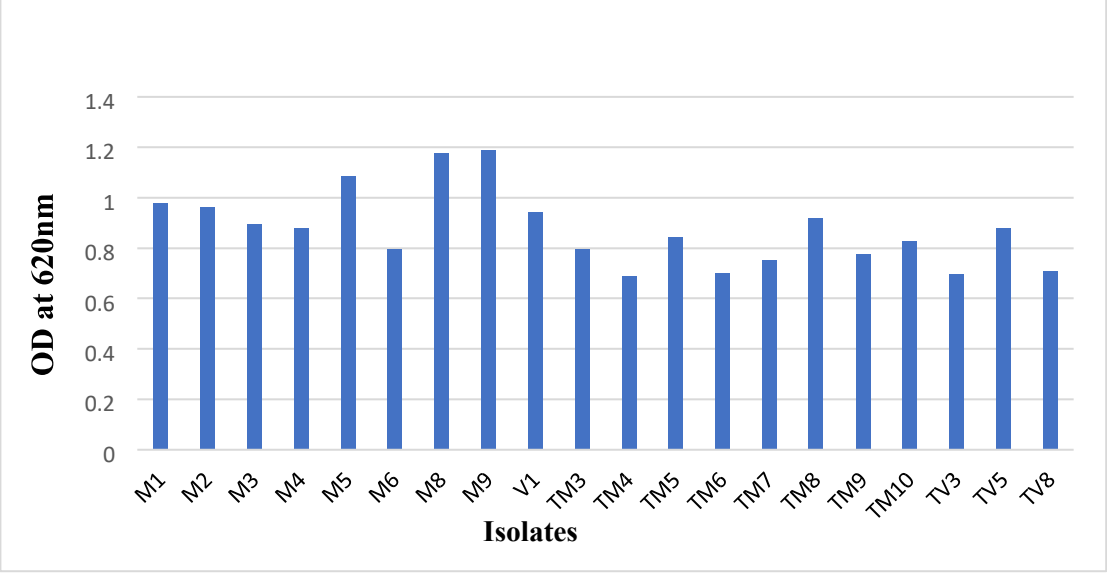


Fig 3.6: Growth of heterotrophic bacterial cultures and *vibrio* spp.

O.D of heterotrophic bacterial isolates and *Vibrio* spp. at 620nm ranged from 0.688 (TM4) to 1.191(M9). These cultures were used for the subsequent experiments.

A)

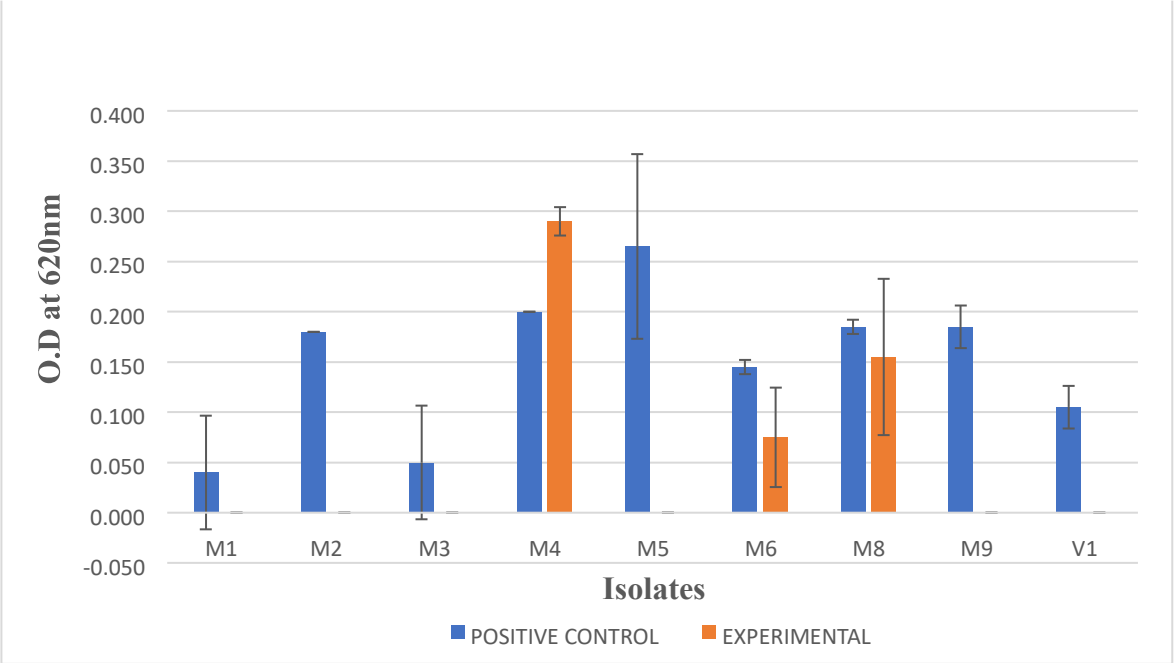


Fig 3.7: Effects of Ampicillin on heterotrophic bacterial culture grown on ZMA (at 620nm).

Compared to the positive control as shown in (**Fig 3.7**), the ampicillin treated heterotrophic bacterial isolates showed total inhibition of bacterial growth in 6 out of 9 cultures tested. Reduction in growth was also observed in M6 and M8. Interestingly ampicillin enhanced growth in isolate M4.

B)

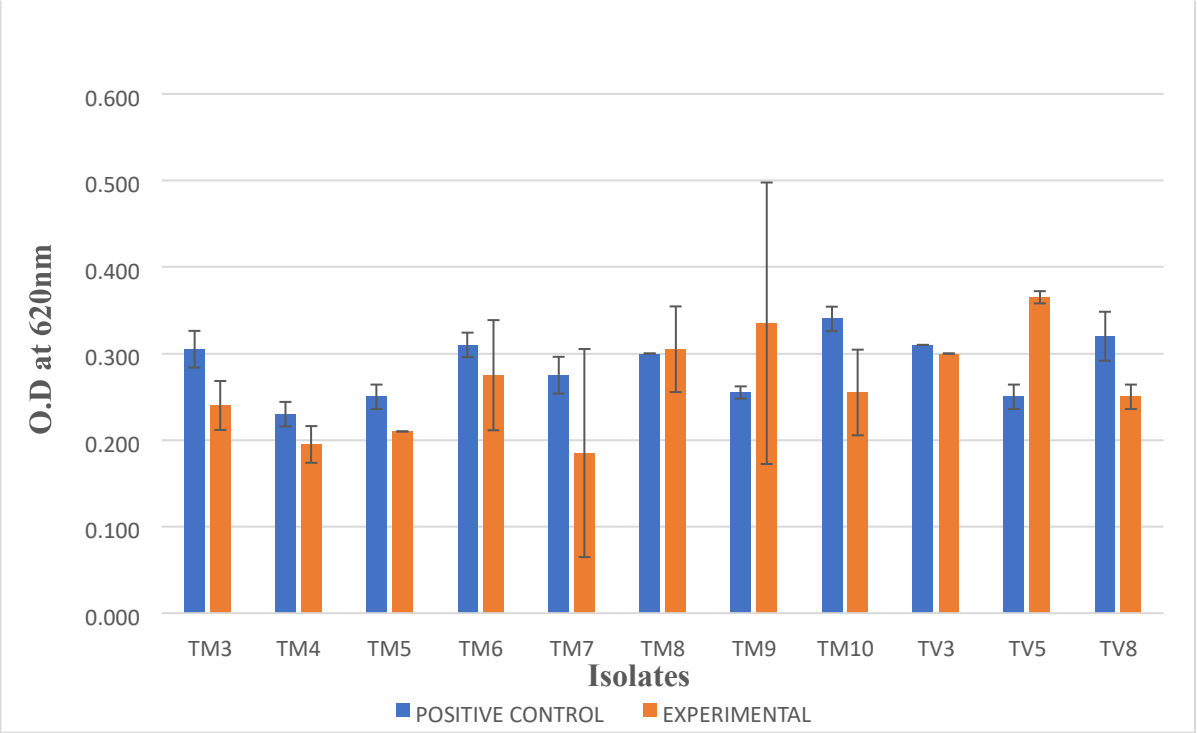


Fig 3.8: Effects of Ampicillin on *Vibrio* spp. grown on TCBS (at 620nm).

Compared to all the positive controls as shown in (**Fig; 3.8**) 9 out of 11 *Vibrio* spp. isolates were inhibited when treated with Ampicillin. Enhanced growth was observed in the presence of Ampicillin in TM8, TM9 and TV5, indicating resistance to ampicillin. Reduction in growth was observed in TM3, TM4, TM5, TM6, TM7, TM10, TV3, TV8. Maximal inhibition was seen in TM7 and minimal inhibition was seen in TV3.

C)

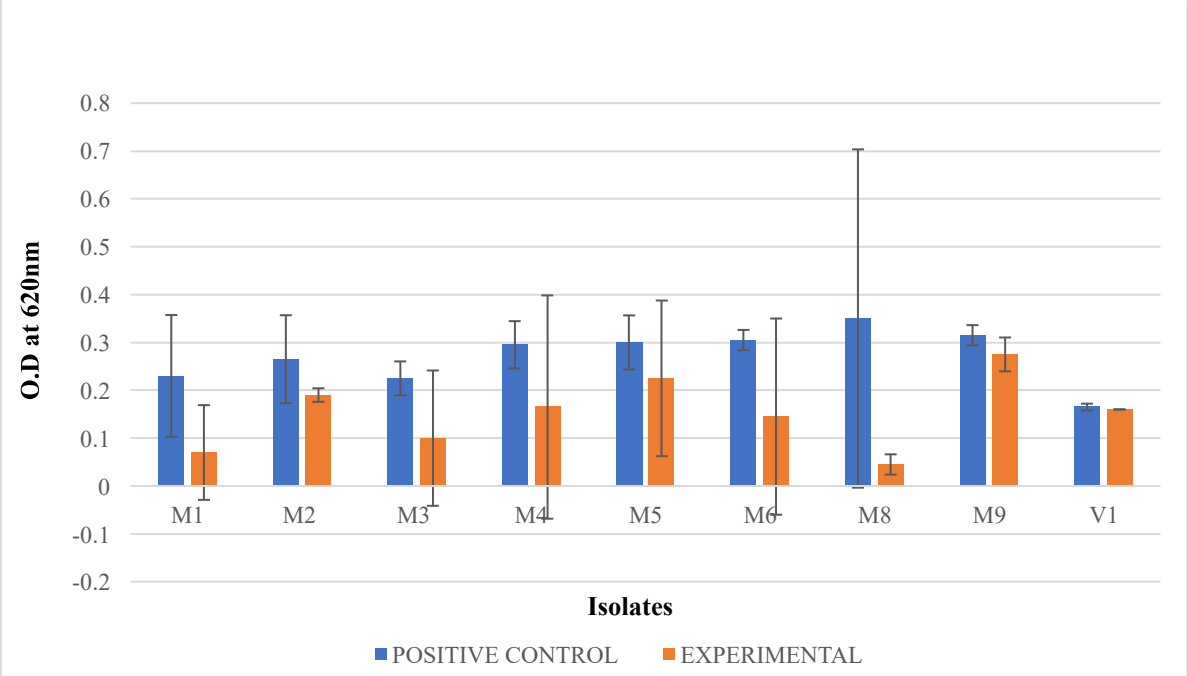


Fig 3.9: Effect of Azithromycin on heterotrophic bacterial culture grown on ZMA (at 620nm).

Compared to the positive controls as shown in (**Fig: 3.9**), the azithromycin treated heterotrophic bacterial culture showed inhibition. Reduction of growth was observed in all the isolates. Maximal inhibition was observed in M8 and minimal inhibition was observed in M9.

D)

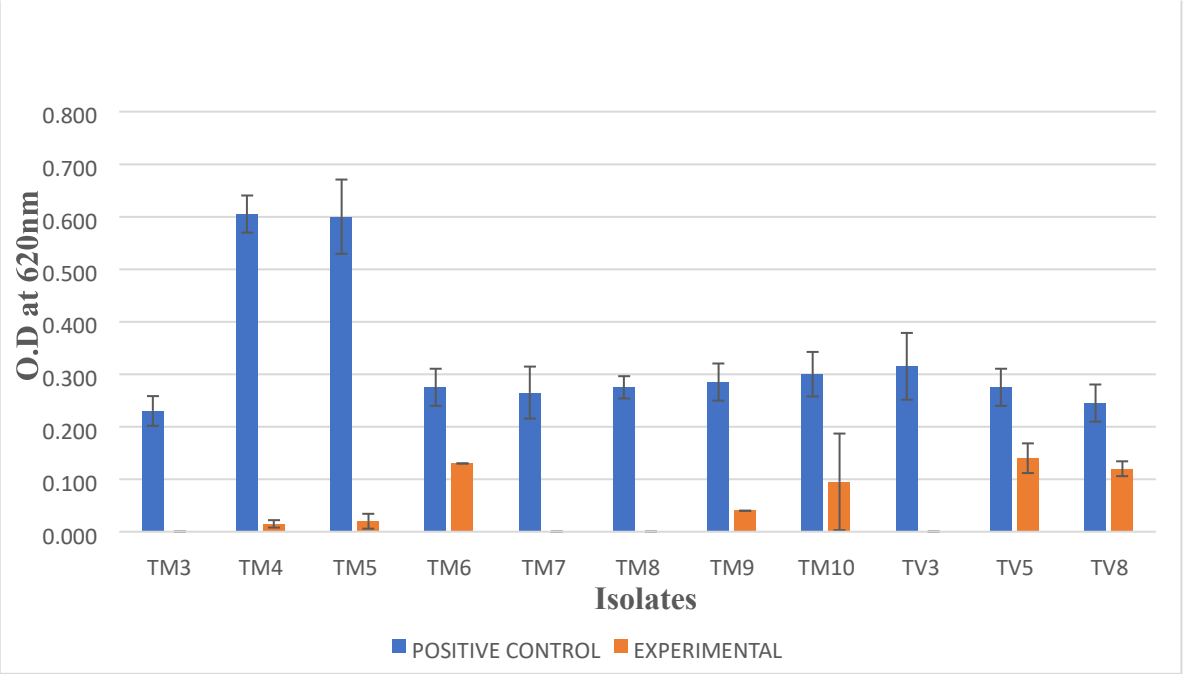


Fig 3.10: Effect of Azithromycin on *Vibrio* spp. grown on TCBS culture (at 620nm).

Compared to all positive controls as shown in (**Fig: 3.10**), the Azithromycin treated *Vibrio* spp. showed total inhibition in isolates TM3, TM7, TM8, TV3. No enhanced growth observed. Reduction in growth was observed in TM4, TM5, TM6, TM9, TM10, TV5, TV8. Maximal inhibition was seen in TM4 whereas the minimal inhibition was observed in TV5.

Comparing the results of effects of ampicillin and azithromycin as shown in (**Fig: 3.7 & 3.9**) on heterotrophic bacterial isolates, ampicillin inhibited 8 of the 9 bacterial isolates tested. In fact, ampicillin showed inhibition to below detectable levels in case of bacterial isolates M1, M2, M3, M5, M9 and V1 (**Fig:3.7**). Azithromycin inhibited 8 of the bacterial isolates, but none below detectable levels (**Fig:3.9**). The lowest inhibition was observed in bacterial isolate V1 which similar growth in presence and absence of azithromycin (**Fig:3.9**). Interestingly ampicillin enhanced growth in isolate

M4, indicating resistance of M4 to ampicillin (**Fig:3.7**). However, culture M4 was inhibited by azithromycin **Fig:3.9**).

Comparing the effect of ampicillin and azithromycin (**Fig 3.8 & 3.10**) on *Vibrio* spp. isolates, the highest inhibition was observed in TM3, TM7, TM8, TV3 showing total inhibition when treated with azithromycin. These 4 isolates showing total inhibition were green in color which may be *Vibrio parahaemolyticus* like organisms. Some isolates like TM8, TM9, TV3 and TV5 also showed enhanced growth indicating resistance to ampicillin. TV3 showed total inhibition when treated with azithromycin but showed resistance when treated with ampicillin. Many of the isolates show very low amount of inhibition which points to resistance to the antibiotics.

The results of this study clearly point to the spread of antibiotic resistance among environmental bacteria. Such an occurrence is a serious environmental concern as reported by (Séveno et al. 2002).

CHAPTER 4

SUMMARY

Antibiotics are antimicrobial agents that work against microorganisms, mainly bacteria. The most prevalent type of antibacterial agent used to treat and prevent bacterial infections is antibiotics (Gould, 2016). Abuse of valuable antibiotics has led to a rapid rise in Antimicrobial Resistance (AMR), making some infections virtually untreatable (Prescott 2014). Studying the antibiotic susceptibility of coastal marine bacterial isolates can also provide valuable information about the prevalence of marine bacterial susceptibility in coastal habitats. Sampling of seawater and sediment sample was done on 2 different stations (Miramar and Anjuna). 20 morphologically distinct colonies from which 9 were heterotrophic bacterial isolates grown on ZMA and 11 were *Vibrio* spp. isolates grown on TCBS. All the isolates were purified in three cycles on nutrient agar and were characterized morphologically by gram staining, motility and catalase activity. All the isolates were tested positive for catalase activity. Effects of antibiotics was tested against Heterotrophic bacteria and *Vibrio* spp. isolates for 2 antibiotics

i.e., Ampicillin and Azithromycin with 50µg/ml which are environmentally relevant concentration. The results were compared to the positive control and discussed. Ampicillin and azithromycin inhibited 8 of the 9 bacterial isolates tested. Ampicillin showed total inhibition of some isolates whereas azithromycin did not. Interestingly ampicillin enhanced growth in isolate M4, indicating resistance of M4 to ampicillin. However, culture M4 was inhibited by azithromycin. The 4 *Vibrio* isolates showing total inhibition to azithromycin were green in color on TCBS agar, and could be *Vibrio parahaemolyticus* like organisms. The results of this study clearly point to the spread of antibiotic resistance among environmental bacteria. Such an occurrence is a serious environmental concern as reported by (Séveno et al. 2002).

APPENDIX

A: Composition of Agar

1) Zobell Marine Agar (ZMA)

INGREDIENTS	GRAMS/LITRE
Peptone	5.00
Yeast Extract	1.00
Ferric citrate	0.10
Sodium chloride	19.45
Magnesium chloride	8.80
Sodium sulphate	3.24
Calcium chloride	1.8
Potassium chloride	0.55
Sodium bicarbonate	0.16
Potassium bromide	0.08
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Agar	15
Final pH (at 25ºC)	7.6 ± 0.2

2) Nutrient Agar (NA)

INGREDIENTS	GRAMS/LITRE
Peptic digest of animal tissue	5.000
Sodium chloride	5.000

Beef extract	1.500
Yeast extract	1.500
Agar	15.00
Distilled water	1000ml
pH (at 25ºC)	7.4 ± 0.2

3) Thiosulphate-Citate-Bile Salts (TCBS) Agar

INGREDIENT	GRAMS/LITRE
Proteose peptone	10.000
Yeast extract	5.000
Sodium thiosulphate	10.000
Sodium citrate	10.000
Bile	8.000
Sucrose	20.000
Sodium chloride	10.000
Ferric citrate	1.000
Bromothymol blue	0.040
Thymol blue	0.040
Agar	15.000
Distilled water	1000ml
pH (at 25ºC)	8.6 ± 0.2

4) Soft (Nutrient) Agar

INGREDIENTS	GRAMS/LITRE
Peptone	5.00
Sodium chloride	5.00
Meat extract	1.50
Yeast	1.50

Agar	2.00
Final pH (at 25ºC)	7 ± 0.2

B: Composition of broth

1) Zobell Marine Broth (ZMB)

INGREDIENTS	GRAMS/LITRE
Peptone	5.00
Yeast Extract	1.00
Ferric citrate	0.10
Sodium chloride	19.45
Magnesium chloride	8.80
Sodium sulphate	3.24
Calcium chloride	1.8
Potassium chloride	0.55
Sodium bicarbonate	0.16
Potassium bromide	0.08
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Final pH (at 25ºC)	7.6 ± 0.2

2) Nutrient Broth (NB)

INGREDIENTS	GRAMS/LITRE
Peptic digest of animal tissue	5.000
Sodium chloride	5.000

Beef extract	1.500
Yeast extract	1.500
Distilled water	1000ml
pH (at 25ºC)	7.4 ± 0.2

C: Composition of reagents

1) Gram staining reagents:

Gram's Crystal Violet (Solution A)

Crystal violet	2g
Ethyl alcohol	20ml

Gram's Crystal Violet (Solution B)

Ammonium oxalate, monohydrate	0.8g
Distilled water	80ml

Solution A and B mixed. Stored for 24 hours before use. The resulting stain is stable.

Gram's Decolourizer

Ethyl alcohol 95%	50ml
Acetone	50ml

Gram's Iodine

Iodine	1g
Potassium iodide	2g
Distilled water	300ml

Safranin,0.5% w/v

Safranin O	0.5g
95% ethyl alcohol	100ml

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