THE COMBINED EFFECTS OF CIPROFLOXACIN AND VITAMIN C ON THE MULTI-DRUG RESISTANT GRAM-NEGATIVE BACTERIA

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fullfillment of the requirements for the degree of
B.Sc in Biotechnology

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Declaration

It is hereby declared that

1. The thesis submitted is our original work while completing the degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

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Abstract

Antibiotic resistance has become a major cause of superinfection and mortality globally. Combination therapy can be used to treat multi-drug-resistant Gram-negative bacteria. Vitamin C can be used in combination with antibiotics. In this study, the effects of combination of an antibiotic and Vitamin C, whether it be synergistic, antagonistic, or additive on multi-drug resistant Gam-negative bacteria were investigated. It was observed that the combination of Ciprofloxacin and Vitamin C showed a synergistic effect on the inhibition of bacterial growth. For *Pseudomonas koreensis*, the mean MIC of the combination was 30 µg/mL (CIP:15 µg/mL, Vitamin C:15 µg/ml) and the mean FICI was 0.47. For *Escherichia fergusonii*, the mean MIC of the combination was 22.5 µg/mL (CIP:7.5 µg/Ml, Vitamin C:15 µg/mL) and the mean FICI was 0.48. For another strain of *P.koreensis*, the mean MIC of the combination was 55 µg/mL (CIP:22.5 µg/Ml, Vitamin C:32.5 µg/mL) and the mean FICI was 0.49 Lastly for *Enterobacter sichuanensis* mean MIC of the combination was 50 µg/mL (CIP:17.5 µg/Ml, Vitamin C:32.5 µg/mL) and the mean FICI was 0.41. Since all the mean FICIs were within 0.5, the combination was found synergistic on these bacteria.

Keywords: Antimicrobial resistance, Gram-negative bacteria, MIC, FICI

Dedication

This is dedicated to our parents for their endless love, sacrifices, and encouragement throughout our pursuit of education. To our best friends for inspiring us and believing in us all along. Lastly, we dedicate this to ourselves for making it through the end.

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List of Acronyms

MDR-GNB	Multi-Drug-Resistant Gram-negative Bacteria
GNB	Gram-Negative Bacteria
MIC	Minimum Inhibitory Concentration
FICI	Fractional Inhibitory Concentration Index
MDR	Multi-Drug Resistant
XDR	Extensively-Drug Resistant
PDR	Pan-Drug Resistant
CAZ	Ceftazidime
CIP	Ciprofloxacin
BHI	Brain-Heart Infusion Broth
MHA	Mueller Hinton Agar
MAC	MacConkey agar
XLD	Xylose Lysine Deoxycholate
MIU	Motility Indole Urea
TSI	Triple Sugar Iron
MSA	Mannitol Salt Agar
NA	Nutrient Agar
AST	Antibiotic Susceptibility Test
MAR	Multiple Antibiotic Resistance
AMR	Antimicrobial Resistance
MLSA	Multilocus Sequence Analysis
ESBL	Extended Spectrum Beta-Lactamase

Chapter 1

Introduction

1.1 Introduction

Antibiotic resistance has been dubbed "the silent tsunami confronting modern medicine." Multidrug resistance in Gram-negative bacteria (MDR-GNB) has become a particularly critical concern for healthcare providers. Gram-negative bacteria (GNB) vary from Gram-positive bacteria in terms of cell wall construction. This causes variances in chemical agent penetration and retention. Gram-negative bacteria have an envelope composed of three major layers: the outer membrane, which contains the (potentially lethal) lipopolysaccharide/endotoxin, the peptidoglycan cell wall with partly cross-linked peptide chains, and the cytoplasmic or inner membrane (Exner et al., 2017).

Since its inception by Migula in 1894, the genus *Pseudomonas* has contained a diverse range of species. *Pseudomonas koreensis* cells are Gram-negative, non-spore-forming rods around 1-2 µm in size with more than one polar flagellum (Kwon et al., 2003). *Pseudomonas spp*. were isolated from the excrement of wild birds in a study. *Pseudomonas spp*. were found in 24 of the 115 samples tested, with 18 having a multiple antibiotic resistance (MAR) index greater than 0.2. The 24 isolates including *Pseudomonas koreensis* tested positive for resistance to Ciprofloxacin and cefepime, both of which are widely used to treat opportunistic *Pseudomonas spp*. infections. Furthermore, their multi-drug resistance profile sheds light on the possible risk of ecosystem pollution. It also emphasizes the necessity of a One Health strategy, such as continual surveillance initiatives that aid in developing a knowledge of how animals, may contribute to and distribute AMR across the ecosystem (C. Rodrigues et al., 2021).

Escherichia fergusonii is a rod-shaped Gram-negative bacterium. *E. fergusonii*, which is closely related to the widely recognized species *Escherichia coli*, was originally isolated from human blood samples. The species is named after William Ferguson, an America microbiologist (Farmer et al., 1985). Some *E. fergusonii* strains are known to be pathogenic. It has been identified as infecting human open wounds and producing bacteremia or urinary tract infections. The bacteria that cause these illnesses are extremely resistant to the drug ampicillin, however, some are also resistant to gentamicin and chloramphenicol (Mahapatra & Mahapatra., 2005). In 2008, an antibiotic-resistant strain of the species was linked to a case of cystitis in a 52-year-old woman (Savini et al., 2008).

Enterobacter sichuanensis is a facultative anaerobe, mesophilic, Gram-negative bacteria isolated from the urine of a patient with chronic renal failure (Podstawka, n.d.). In hospitalized patients, *Enterobacter spp*. Causes diseases like urinary tract infections (UTI), pneumonia, and bloodborne infections. Enterobacter cloacae complex (ECC) species are ubiquitous in the environment and are generally recognized as opportunistic infections. ECCs such as *E. hormaechei, E. sichuanensis, E. asburiae, E. kobei,* and *E. roggenkampii* have been a worldwide health issue in recent decades due to widespread antibiotic resistance and novel multi-drug resistance. They are naturally resistant to beta-lactam antibiotics, which include ampicillin, amoxicillin, and first-generation cephalosporins (Uchida et al., 2020).

Combination therapy, such as β-lactam coupled with β-lactamase inhibitors or combination antibiotics, can be used to treat MDR microorganisms. Antibiotics + non-antibiotic medications or antibiotics plus other antibiotics are examples of combinations (Hagihara et al., 2012). As various combination medications have been licensed in additional countries in recent years, the treatment possibilities have broadened. However, only a few guidelines treat and endorse these

alternatives, and the recommendations are based on low-quality research (Vardakas et al., 2018). Vitamin C which is also called Ascorbic acid can be used in combination therapy alongside antibiotics. Ascorbic acid contains antibacterial and antiviral properties, which are required to enhance immunological function (activating phagocytic leukocytes), and lower the intensity and duration of illness, as well as the inflammation caused by bacterial infection. Large doses of ascorbic acid were stated to function synergistically with appropriate antibiotics when used to treat bacterial infections, greatly expanding the medications' action range (Qushawi1 & Al-Ruaby., 2021).

In this reseach, combination therapy was studied between an antibiotic and Vitamin C and tested if they were synergistic, antagonistic, or additive. It was found out that although the combination of Ceftazidime and Vitamin C is antagonistic, the combination of Ciprofloxacin and Vitamin C is synergistic on the multi-drug resistant Gram-negative bacteria. (MDR GNB).

1.2 Character and Morphology

The CM-01 is a strain of *P. koreensis* whose genome is 6,171,880 bp in length and has a G+C content of 60.5%. In addition, the genome sequence contains 5538 protein-coding genes, 3 rRNA genes, 54 tRNAs, and no plasmids. Aside from these, the *P. koreensis* CM-01 genome projected 39 interspersed repeat and 141 tandem repeat sequences, 6 prophages, 51 genomic islands, 94 insertion sequences, 4 clustered regularly interspaced short palindromic repeats, 5 antibiotic-resistant genes, and 150 virulence genes. Furthermore, the assembled CM-01 genome was successfully annotated against the databases Cluster of Orthologous Groups of Proteins, Gene Ontology, and Kyoto Encyclopedia of Genes and Genome Pathways. A comparison of CM-01 with three typical strains of *P. koreensis* found that 92% of orthologous

clusters were conserved throughout these four genomes, with only the CM-01 strain containing unique pathogenicity and virulence components (Kho et al., 2022).

E. fergusonii is a Gram-negative, rod-shaped member of the *Enterobacteriaceae* family. It is a non-spore-forming, motile bacterium with a diameter of 0.8-1.5 µm, lengths ranging from 2 to 5 µm, and peritrichous flagellation. *E. fergusonii* are facultative anaerobes that may get energy from organic oxidation and reduction processes. *E. fergusonii* strains are catalase-positive and oxidase negative (Savini et al., 2008). The *Enterobacteriaceae* are bacilli (rod-shaped bacteria) that range in size from 1 to 5 µm. On blood agar, they normally show as medium to large grey colonies, while some can express colors. Most have several flagella that they utilize to move about, although a few genera are nonmotile. The majority of *Enterobacteriaceae* feature peritrichous, type I fimbriae that aid in bacterial cell attachment to their hosts. They do not produce spores (Edwards & Ewing., 1972).

Enterobacter sichuanensis is a Gram-negative bacterium that is facultatively anaerobic, and mesophilic, and forms circular colonies. An E. fergusonii strain known as SGAir0282 had a single contig with a 4.71 Mb genome assembly and a G+C content of 55.5%. Plasmids were not discovered during the assembly. The Prokaryotic Genome Annotation Pipeline (PGAP) of the NCBI predicted that the genome would contain 25 rRNA genes, 83 tRNA genes, and 4371 coding genes. Streptothricin acetyltransferase (SatA), fosfomycin resistance protein (FosA), and metal-dependent hydrolases of the beta-lactamase subfamily I (BLI) were among the genes identified that were related to antibiotic resistance. (Uchida et al., 2020)

1.3 Emergence of Antibiotic-Resistant Bacteria

Antibiotic exposure has been identified as the single most critical factor driving the establishment and spread of antibiotic resistance. This viewpoint stresses the role of natural

(Darwinian) selection in the evolution of resistance, arguing that antibiotic-resistant organisms survive and procreate whereas susceptible counterparts fall extinct. In fact, though, circumstances are more complex. Antibiotic resistance should be viewed as the formation of an aberrant resistance characteristic in a specific microorganism, most commonly a bacterial pathogen. However, emergence is typically noticed as a phenomenon only after the new resistance characteristic is present at a sufficiently high frequency in an organism, and therefore the first incidence of resistance may often remain mysterious (Cantón & Morosini., 2011).

Pseudomonas koreensis, a novel Gram-negative bacterium, was isolated and reported for the first time from Korean agricultural soil. CRS05-R5 was discovered in the rice rhizosphere of Heilongjiang province in 2003 (Xie et al., 2003). Scientists recently sequenced CRS05-R5's 16S rRNA sequence and constructed a phylogenetic tree. As a result, they determined that CRS05-R5 should be categorized as *P. koreensis*. However, only one genome (D26) was sequenced, and no extensive investigation of this species was conducted. In this case, they performed whole-genome sequencing on CRS05-R5 to uncover the likely mechanism behind its antagonistic ability (Lin et al., 2016).

E. fergusonii, formerly known as enteric Group 10, is a rare but developing animal and human disease-causing bacteria named after the American scientist William H. Ferguson. The clinical relevance of *E. fergusonii* was unknown at the time of discovery, but it has subsequently been isolated mostly from instances of wound infection, urinary tract infection, bacteremia, diarrhea, pancreatic cancer, endophthalmitis, and pleuritis in humans. *E. fergusonii* has been discovered to produce salmonellosis-like illness in sheep and cattle, with clinical symptoms such as abortion, scour, and mastitis (Bain and Green., 1999). A multi-drug resistant isolate of *E. fergusonii* from a pig in Korea showed positive in a PCR for heat-stable and heat-labile toxins

(STa, LT), as well as fimbriae adhesins of *E. coli* known to be implicated in pig enteric illness. (Rayamajhi et al., 2011).

The genus *Enterobacter* belongs to the ESKAPE group, which includes the most common resistant bacterial infections. This group, first identified in 1960, has proven to be increasingly complicated as a result of the exponential expansion of phenotypic and genotypic approaches. The *Enterobacter* genus now contains 22 species. These organisms have been identified in the environment as opportunistic pathogens in plants, animals, and humans (Davin-Regli et al., 2019).*Enterobacter chengduensis* and *E. sichuanensis* were identified in China from human blood samples and urine, respectively, and were thought to be novel species based on phenotypic traits and phylogenetic analysis using Multilocus Sequence Analysis (MLSA) (Singh et al., 2018).

1.4 Mechanism of Antibiotic-Resistant Bacteria

Antimicrobial resistance is caused by three primary mechanisms: enzymatic breakdown of antibacterial medications, changes in bacterial proteins that are antimicrobial targets, and modifications in membrane permeability to antibiotics. Antibiotic resistance can be transmitted via plasmids or maintained on the bacterial chromosome (*Mechanisms of Bacterial Resistance to Antibiotics.*, 1991). The mechanism of antimicrobial resistance in GNB arises from the expression of antibiotic-inactivating enzymes and non-enzymatic paths that may result from increasing intrinsic resistance due to chromosomal gene mutations (such as boosting the production of enzymes that inactivate antibiotics, efflux pumps, permeability, or target alterations) or accumulated by the transfer of genetic components that are mobile, containing resistance genes such as plasmids expressing β-lactamases, aminotransferases, and so on (Breijyeh et al., 2020).

Pseudomonas spp. resistance strategies are diverse and assisted by their genetic flexibility, including multi-drug efflux systems, outer membrane protein loss, target mutations, and enzyme synthesis (C. Rodrigues et al., 2021).

A Chinese investigation discovered that the predominance of *E. fergusonii* isolates was diversified, with significant levels of antibiotic resistance. The colistin resistance gene mcr-1 was found in 18.8% of *E. fergusonii* isolates. As a reservoir of mcr-1, E. fergusonii may aid in the evolution of colistin resistance. Furthermore, it was revealed that *E. fergusonii* had a variety of Anti-microbial resistant (AMR) genes, including several well-known genes such as extended-spectrum beta-lactamases (ESBLs) (12), suggesting that *E. fergusonii* might be a significant reservoir of AMR genes. In 2018, a mcr-1-positive E. fergusonii was discovered in Guangdong, China. Following that, in 2019, an E. fergusonii plasmid with a complete sequence harboring both mcr-1 and ESBLs was isolated from chicken feces in Zhejiang, China, implying that *E. fergusonii* may play an important role in mcr-1 transmission and pose a serious threat to clinical infection treatment (Tang et al., 2022).

The fundamental mechanism of beta-lactam resistance in *Enterobacteriaceae* is the synthesis of beta-lactamase. These very diverse enzymes hydrolyze beta-lactams in the periplasmic region, avoiding penicillin-binding protein inhibition (Ruppé et al., 2015).

1.5 About Ciprofloxacin

Ciprofloxacin, belonging to the fluoroquinolone drug class, demonstrates effectiveness in treating a wide range of bacterial infections caused by both Gram-negative bacteria, including *Pseudomonas, Enterobacter, Klebsiella , and Escherichia coli,* and Gram-positive bacteria, such as *Staphylococcus aureus*. This antibiotic exerts its action by inhibiting the activities of DNA-gyrase and DNA topoisomerase, crucial enzymes involved in DNA replication, recombination, and repair processes (Shariati et al., 2023).

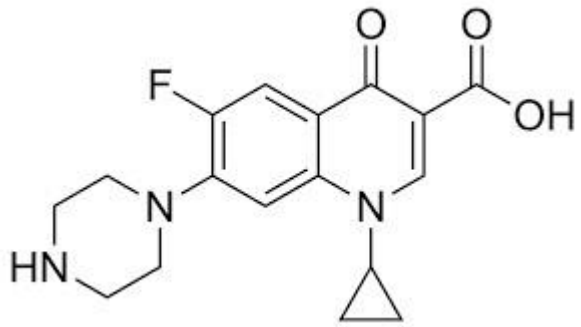


Figure 1: Structure of Ciprofloxacin

In 1983, Bayer A.G. patented Ciprofloxacin, and four years later, in 1987, the United States Food and Drug Administration (USFDA) granted its approval. Ciprofloxacin is an FDA-approved antibiotic with a broad spectrum of applications. It is used to treat various infections, including urinary tract infections, sexually transmitted infections (such as gonorrhea and chancroid), skin and soft tissue infections, bone and joint infections, prostatitis, pneumonia, typhoid fever, gastrointestinal infections, lower respiratory tract infections, inhalation anthrax (post-exposure prophylaxis), plague, salmonellosis, and acute bacterial exacerbation of chronic bronchitis (*Ciprofloxacin.*, 2023).

Ciprofloxacin stands out as a readily accessible and cost-effective antibiotic in Bangladesh, with widespread availability across numerous pharmacies. This multipurpose antibiotic caters to the needs of a densely populated developing nation like Bangladesh. Its affordability and versatile applications make it an indispensable resource. Leveraging the potential of Ciprofloxacin in the battle against antibiotic-resistant bacteria could potentially lead to a revolutionary breakthrough.

1.6 About Vitamin-C

Vitamin C, also known as ascorbic acid, is widely recognized for its role in supporting the immune system and promoting overall health. Vitamin C performs well both in vitro and in

vivo as a bactericidal and anti-biofilm agent, either alone or in combination with antibiotics.. However, emerging research suggests that Vitamin C might also play a role in modulating bacterial susceptibility, particularly against certain Gram-negative pathogens such as *Pseudomonas, E Coli* and *Enterobacter*.

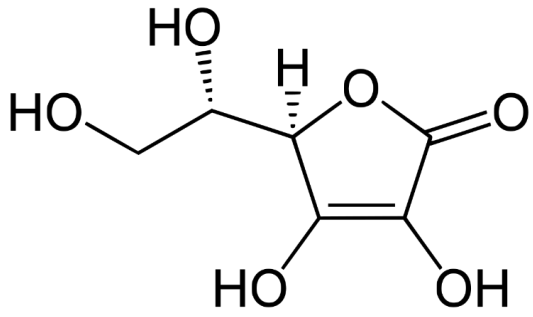


Figure 2: Structure of Vitamin C

The combination of antibiotics and Vitamin C (ascorbic acid) has been investigated for its potential effects on Gram-negative bacteria. Several studies have explored the interactions between antibiotics and Vitamin C in the context of bacterial infections, particularly those caused by Gram-negative bacteria. Research has suggested that the combination of antibiotics and Vitamin C may have synergistic effects in combating Gram-negative bacteria. Vitamin C's antioxidant properties can help reduce oxidative stress and inflammation, potentially enhancing the effectiveness of antibiotics in eradicating these bacterial infections (Li et al., 2018). For example: a series of studies have started to shed light on the captivating interplay between Vitamin C and bacterial susceptibility. It was discovered that supplementing with Vitamin C increased the vulnerability of *Pseudomonas spp* to particular antibiotics through its impact on efflux mechanisms, notably the MexXY system. This not only highlighted the possibility of boosting antibiotic effectiveness but also provided valuable insights into the mechanisms behind bacterial resistance (Tanaka et al., 2018). Collectively, these studies shed light on the

multifaceted relationship between Vitamin C and bacterial susceptibility, offering a promising avenue for further research into novel strategies to combat multi-drug-resistant pathogens.

1.7 MDR, XDR, PDR

When discussing bacteria that remain unaffected by antibiotics, scientists commonly employ terms such as MDR (Multi-Drug Resistant), XDR (Extensively Drug Resistant), and PDR (Pan-Drug Resistant). These terminologies play a pivotal role in characterizing organisms that pose formidable challenges in terms of treatment within the context of today's advanced medical landscape. These three distinct categories serve as valuable tools for researchers, facilitating the categorization and classification of these resilient microorganisms.

MDR means "multidrug resistance." Bacteria are called MDR if they don't respond to at least one important antibiotic of three classes (Rex., 2023). Labs test bacteria to see which antibiotics they can resist. The commonly accepted definition is when an organism, whether it's Gram-positive or Gram-negative, shows resistance to three or more classes of antimicrobial agents, it is identified as MDR (Magiorakos., et al, 2012)

The term "extensively drug-resistant," known as XDR, pertains to a category of bacteria that have acquired resistance to a broad spectrum of antibiotics, encompassing both primary and secondary treatment options. XDR bacteria commonly show resistance to several categories of antibiotics, significantly constricting the therapeutic choices available to healthcare providers. XDR bacteria's resistance mechanism relies on a range of genetic mutations that grant them safeguards against antibiotics. These mutations can arise naturally or result from exposure to antibiotics. There are two approaches to defining XDR. The first approach involves considering the various classes or subclasses of drugs an organism is resistant to. The second approach

focuses on the number of essential antimicrobial agents that the organism is resistant to, requiring this number to exceed one (Magiorakos., et al., 2012).

PDR, which stands for pan-drug-resistant, designates a group of bacteria that have developed resistance against virtually all accessible antibiotics, even those considered as last-resort options (Magiorakos, et al., 2012). The acronym PDR, derived from "pan drug-resistant," encapsulates its meaning in the prefix "pan-," originating from ancient Greek and signifying "all" or "whole". As elucidated in Dorland's Illustrated Medical Dictionary, this prefix has been extensively employed in the realm of biomedical terminology to connote the comprehensive inclusion of all elements or aspects within an organism. In this context, the term "pan resistance" or "pan drug resistance" (PDR) explicitly denotes resistance encompassing all antibiotics, leaving no room for interpretation otherwise (Falagas & Karageorgopoulos., 2008).

1.8 Objectives of the Study

- Developing a novel, long-term answer to the growing problem of multi-drug-resistant Gram-negative bacteria like *Pseudomonas koreensis, Escherichia fergusonii, and Enterobacter sichuanensis* .
- Determining the effectiveness of Ciprofloxacin when combined with Vitamin C in the inhibition of growth of multi-drug-resistant Gram-negative bacteria *Pseudomonas koreensis, Escherichia fergusonii,* and *Enterobacter sichuanensis*.

Chapter 2

Materials and Methods

2.1 Methodology

The experiment was conducted at the MNS Thesis Lab of BRAC University. Prospective studies, including trials, were done to determine the effects of the combination of Ciprofloxacin and Vitamin C on the multi-drug resistant Gram-negative bacteria.

Samples were collected from three different bacterial genera: *Pseudomonas, Escherichia, and Enterobacter*. These samples were collected using Nutrient agar slants from Mohakhali's TB Hospital in Dhaka. First, the samples were isolated and subsequently cultured on nutrient agar. Following that, an Antibiogram was conducted, which involved the use of 15 different antibiotics from nine separate classes on Mueller-Hinton Agar (MHA). Antibiotics are commonly prescribed by physicians to combat bacterial infections. The results of the Antibiogram indicated that the samples displayed resistance to 96% of the key antibiotics, and all of the strains exhibited Multi-drug Resistance (MDR).

From the list of antibiotics against which bacteria showed high resistance, Ceftazidime and Ciprofloxacin were chosen for testing in conjunction with Vitamin C. First, the individual Minimum Inhibitory Concentrations (MIC) for Ceftazidime were determined. The following stage involved doing MIC tests with various dosages of a Vitamin C and ceftazidime combination. The initial test results showed that Ceftazidime and Vitamin C combined were ineffective since the FIC Index was over 0.5. Ciprofloxacin, another antibiotic, was also selected.The individual MIC of Ciprofloxacin, individual MIC of Vitamin C, and the MIC of a combination of Ciprofloxacin and Vitamin C showed effective results as the FIC index was

within 0.5. Thus, the combination of Ciprofloxacin and Vitamin C was chosen as the treatment regimen for *Pseudomonas*, *Escherichia*, and *Enterobacter* isolates for the research.

2.2 Sample Collection

The microbiology department at the National Institute of Diseases of the Chest and Hospital (NIDCH) has provided clinical samples belonging to the *Pseudomonas, Escherichia*, and *Enterobacter* genera. The samples were collected in nutrient agar tubes. Subculture on nutrient agar was performed on the samples. When it arrived at the BRAC University lab, it was carefully moved into the incubator and kept there for 24 hours at 37°C. After the initial 24 hours of incubation, the samples were sub cultured once again on the nutrient agar dish using the streak plate method, and the incubation was carried out for 24 hours at 37°C. The samples were subcultured until a single colony was obtained from each. After the initial growth, each sample was also examined for purity and identification using Gram staining and several biochemical techniques.

Therefore, the samples were streaked on agar plates with a particular medium for continuous cultures, such as MacConkey agar plates for *Escherichia* and *Enterobacter*, and Cetrimide agar plates for *Pseudomonas*. This precaution was made to avoid contamination. The samples were also stabbed into T1N1 agar, and after incubation paraffin oil was added on top and the vials were wrapped with parafilm and thus kept at rtp as stock.

2.3 Biochemical Tests

2.3.1 Gram Staining

Gram staining is the most significant differential stain used in bacteriology to distinguish between two types of bacteria: Gram-positive and Gram-negative, making it an important tool in the process of identifying and distinguishing microorganisms.

First, clean, grease-free glass slides were labeled, one for each species. After that, an inoculation loop was sterilized by exposing it to a flame that was utilized to eradicate organisms from the culture. A swath of distilled water was placed on the slide. Following that, one of the types of organisms (A/B) was removed from the culture plate, and the smear was created by thoroughly spinning the loop holding the culture on the slide. The material on a slide was air-dried, then heat-fixed by passing it over a flame and allowed to cool before staining. The slide was then smeared with crystal violet and set aside for 1 minute. The slide was gently washed with tap water while holding it at 45°. The smear was moistened with Gram iodine mordant for 1 minute. Again, the slide was gently washed with tap water holding it at 45°. Decolorized for 5-15 seconds with 95% ethanol. To avoid over-decolorization, drop-by-drop reagent was added until crystal violet did not wash away from the smear. Gently the slide was washed with tap water while holding it at 45. a counterstain with safranin was added and kept for 1 minute. Gently the slide was washed with tap water while holding it at 45°. Allowed to dry before putting on the cover slip. Examined with a microscope.

2.3.2 Oxidase Test

This biochemical test identifies the presence of the enzyme cytochrome oxidase. A 1% solution of tetramethyl-p-phenylene-diamine dihydrochloride was applied to Whatman's filter paper, which was then dried. After that, the paper was put in a petri dish and coated with distilled water. An autoclaved cotton swab was used to pick up the bacterial colony to be examined, which was then spread across the damp region. After 15 minutes, the colony was checked for the appearance of a strong purple color.

2.3.3 Catalase Test

This test shows that catalase, an enzyme that facilitates the oxygen release from hydrogen peroxide (H_2O_2), is present. A glass slide was loaded with a loopful of the sample culture, and one drop of 3% hydrogen peroxide was added. The slides were then checked for any quick bubbling effect.

2.3.4 Motility Indole Urea Test

The Motility Indole Urea (MIU) test helps identify Gram-negative bacteria, particularly those belonging to the *Enterobacteriaceae* family. Three tests in one tube aid in differentiating the organisms based on their motility, synthesis of urease, and indole. MIU agar was made and poured into test tubes followed by autoclaving them. After the medium had cooled, a 40% urea solution was added, thoroughly mixed, and then allowed to crystallize upright. A few colonies were stabbed directly in the agar to inoculate it. At 37 °C, the cultures were incubated for 24 hours. Following that, medium color changes were checked to see whether growth had occurred.

2.3.5 Triple Sugar Iron Test

Triple Sugar Iron (TSI) medium is a differential medium that can distinguish between several Gram-negative enteric bacteria based on their physiological ability (or lack thereof) to metabolize lactose and/or sucrose, conduct fermentation to produce acid, produce gas during the fermentation, and generate H_2S. After the TSI media was made, it was autoclaved and dried in a slant position. Then the test tubes were inoculated with 24-hour culture by stabbing and streaking the slants. After incubating them at 37℃ for 24 hours, their color change was observed to determine the growth of bacteria.

2.3.6 Methyl Red Test

The methyl red (MR) test measures the amount of acid produced during the fermentation of glucose and the maintenance of conditions that keep an old culture's pH below 4.5, as indicated by a change in the color of the methyl red indicator that is added after the incubation period. The MRVP broth was prepared and autoclaved followed by inoculation with organisms from an 18–24-hour pure culture. Then the media was incubated aerobically at 37°C for 48 hours. Following the incubation, 2-3 drops of the methyl-red indicator were added to the aliquot, and the color change was observed and recorded.

2.3.7 Voges Proskauer Test

To find out whether an organism makes acetyl methyl carbinol from glucose fermentation, the Voges-Proskauer (VP) test is carried out. After preparing and autoclaving the MRVP broth, it was inoculated with organisms obtained from an 18–24 hours pure culture followed by 48 hours of aerobic incubation at 37℃. After the incubation period, the broth was stirred well to aerate before adding 6 drops of 5% alpha-naphthol (Barritt's reagent A). Then 2 drops of Barritt's reagent B were added and thoroughly mixed to aerate the mixture. Within 30 minutes, it was observed for a pink-red tint near the surface.

2.3.8 Indole Test

This experiment shows that certain bacteria can break down the accumulating tryptophan into the amino acid indole. The indole synthesis test is critical for detecting enterobacteria. Sterile test tubes with 4 mL of tryptophan broth were taken and autoclaved. Using an aseptic technique, the growth from an 18 to 24-hour culture was used to inoculate the tube. For 48 hours, the tube was incubated at 37°C. Kovac's

reagent was applied to the broth culture at a volume of 0.5 mL. It was checked to see whether there was a ring over it.

2.3.9 Nitrate Reduction Test

The nitrate reduction test evaluates the synthesis of an enzyme called nitrate reductase, which results in nitrate reduction. Nitrate broth was made and autoclaved in test tubes. Under the laminar hood, a loop filled with bacterial culture was inoculated into the broth. The cultures were incubated at 37 °C for 24 hours. Following incubation, nitrite reagent A and reagent B were introduced in a 1:1 ratio and the color change was observed.

2.4 Collection of Antibiotics

The 2 antibiotics used in the MIC determination process were:

1. Ceftazidime
2. Ciprofloxacin

Ceftazidime: Ceftazidime, a beta-lactam antibiotic of the semi-synthetic broad-spectrum class was utilized. The Ceftazidime for injection, formulated by Aristropharm Ltd, is a sterile, powdered mixture containing Ceftazidime pentahydrate and sodium carbonate (118 mg/g of Ceftazidime). Ceftazidime kills bacteria by stopping them from building their cell walls. It can work against a wide range of Gram-negative bacteria, even those resistant to gentamicin and similar antibiotics. It also works against some Gram-positive bacteria..

Ciprofloxacin: Ciprosin eye drop of Square Pharmaceuticals Ltd was utilized. Ciprosin 0.3% Eye Drops is an antibiotic belonging to the fluoroquinolone family, designed to combat bacterial infections. Every 5 mL of this contains 3 mg of Ciprofloxacin. So, a 0.3% solution was created. Its efficacy extends to treating various severe bacterial infections such as

pneumonia, respiratory and urinary tract infections, gonorrhea, anthrax, gastroenteritis, sinus infections, bone infections, skin infections, and joint infections.

Table 1: List of Antibiotics used

Number	Name of Antibiotics	Class
1.	Amikacin	Aminoglycosides
2.	Ampicillin	Penicillin
3.	Azithromycin	Macrolides
4.	Cefepime	Beta-lactam
5.	Ceftazidime	Beta-lactam
6.	Ciprofloxacin	Fluoroquinolone
7.	Ceftriaxone	Cephalosporin
8.	Erythromycin	Macrolide
9.	Gentamicin	Aminoglycoside
10.	Imipenem	Carbapenems
11.	Levofloxacin	Fluoroquinolones
12.	Moxifloxacin	Fluoroquinolones
13.	Norfloxacin	Quinolone
14.	Penicillin	Beta-lactam
15.	Tetracycline	Tetracycline

2.5 Preparation of Media

2.5.1 Nutrient Agar

Initially, the organisms were cultivated on nutrient agar. The organisms were cultured on nutrient agar by dissolving 28 g of nutrient agar powder in 1 L of distilled water, and heated until the agar melted. After autoclaving the solution for 15 minutes at 121°C, it was allowed to

cool down. The cooled agar was then poured onto Petri dishes inside a laminar flow cabinet and left to solidify.

2.5.2 MacConkey Agar

MacConkey agar was used for the isolation of Gram-negative enteric bacteria and the differentiation of lactose fermenting from lactose non-fermenting Gram-negative bacteria. MacConkey agar utilizes lactose as the differentiating factor. When lactose is fermented by bacteria, it produces acidic byproducts, causing the pH indicator, neutral red, to turn into a bright pink-red color. As a consequence, bacteria like *Escherichia coli*, capable of lactose fermentation, form colonies that exhibit a distinctive bright pink-red color.

MacConkey agar was chosen to differentiate between different members of the *Enterobacteriaceae* family as it is a selective medium that ensures sample purity and prevents contamination. To prepare the MacConkey media, 49.53g of MacConkey agar powder was dissolved in 1 liter of distilled water. The mixture was heated until the agar melted, and then it was autoclaved at 121°C for 15 minutes. After autoclaving, the medium was cooled down in the laminar flow cabinet before being poured into Petri dishes. The solidified media were then stored in the refrigerator until needed.

2.5.3 Cetrimide Agar

Cetrimide Agar is used for the selective isolation and presumptive identification of *Pseudomonas spp*.To prepare the medium, 45.3 Grams of the medium and 10 mL of glycerol were suspended in one liter of distilled water. The mixture was heated with frequent agitation and boiled for one minute to ensure complete dissolution of the medium. Afterward, the solution was autoclaved at 121°C for 15 minutes to achieve sterilization. Once autoclaved, the medium was thoroughly mixed and poured into sterile Petri plates.

2.5.4 Mannitol Salt Agar

MSA (Mannitol Salt Agar) is a selective and differential growth medium used to isolate and identify pathogenic *Staphylococcus* species, particularly *Staphylococcus aureus*. To prepare this medium, MSA powder was dissolved in one liter of boiled distilled water. The solution was then autoclaved at 121°C for 15 minutes after a short cooling period. Once autoclaved, the MSA medium was poured into sterile Petri dishes within a laminar flow cabinet and allowed to solidify.

2.5.5 Xylose Lysine Deoxycholate

XLD (Xylose Lysine Deoxycholate) is a selective and differential growth medium used to isolate and differentiate *Salmonella* and *Shigella* species, especially from fecal samples. To prepare XLD, the XLD powder was dissolved in one liter of boiled distilled water. The solution was then autoclaved at 121°C for 15 minutes and allowed to cool briefly. After autoclaving, the sterile XLD medium was poured into Petri dishes inside a laminar flow cabinet and left to solidify.

2.5.6 Brain Heart Infusion Broth

The MIC determination procedure was conducted in BHI (Brain Heart Infusion) broth. To prepare the broth, 37 g of BHI powder was dissolved in one liter of distilled water without additional heating. The resulting solution was carefully transferred into individual test tubes using a glass pipette, with each test tube containing 5 mL of the BHI broth. The test tubes, inside a beaker, were sterilized by autoclaving at 121°C for 15 minutes. After sterilization, the test tubes were inoculated with bacterial suspension and antibiotic and (or) Vitamin C.

2.5.7 Mueller Hinton Agar

MHA (Mueller-Hinton Agar) was used as the growth medium during the antibiogram procedure. One of the advantageous aspects of MHA is that it is a non-selective medium, allowing any type of organism to grow on it. Additionally, MHA agar's soft consistency allows rapid antibiotic diffusion in the disc diffusion method, enabling easy measurement of bacterial susceptibility. To prepare the MHA, 38g of MHA powder was dissolved in one liter of distilled water. The solution was then boiled followed by autoclaved at 121℃ for 15 minutes. Once autoclaved, the medium was poured into Petri dishes within the laminar flow cabinet and left to solidify.

2.6 Physiological Saline Preparation

Biological suspensions of the bacteria were prepared using physiological saline. To achieve the required concentration of 0.9% sodium chloride, it was dissolved in 100 mL of distilled water. Any excess would lead to an overly alkaline environment, resulting in the death of the bacteria. Subsequently, 10 mL of the saline solution was added to each test tube using a glass pipette. The test tubes were then autoclaved at 121°C for 15 minutes and left at room temperature afterward.

2.7 Preparation of Bacterial Suspension

The bacterial suspension was prepared using physiological saline. Two to three colonies of bacteria were taken from a young culture using a sterile loop and dissolved in saline. To ensure the complete dissolution of the bacteria, the solution was vortexed. Subsequently, it was compared to solutions using the MacFarland standard 0.5 for further analysis.

2.8 Preparation of Vitamin C

The Vitamin C solution was prepared by dissolving ascorbic acid in the distilled water. In the experiment, 3g of ascorbic acid was measured and dissolved into 25 mL of autoclaved distilled

water. The mixture was then stirred thoroughly to ensure the complete dissolution of the ascorbic acid in the water. To further refine the solution and eliminate any undissolved particles or impurities, a filtration apparatus was set up using a membrane filter funnel and a syringe. The prepared Vitamin C solution was then carefully transferred using a syringe through a membrane filter funnel. The filtrate was collected in an empty, autoclaved beaker. This precise filtration process effectively yielded a clear and pure Vitamin C solution.

2.9 Antibiotic Susceptibility Test (AST)

In this experiment, an antibiogram study was conducted to assess the antibiotic susceptibility of the collected samples of bacteria. The disc diffusion technique was employed for this purpose. Initially, bacterial suspensions were evenly spread over Mueller Hinton Agar (MHA) plates using sterile cotton swabs. Each plate received seven antibiotic disks, and 17 MHA plates were used for each collected sample. The plates were thereafter placed in the incubator for 24 hours at 37°C. After the 24-hour incubation period, the clear zones around each antibiotic disk were measured using a ruler, and they were compared to established norms. This comparison enabled the determination of which samples were susceptible to specific antibiotics and which antibiotics exhibited resistance. The antibiogram analysis provided valuable insights into the antibiotic sensitivity patterns of *Pseudomonas koreensis*, *Enterobacter sichuanensis and Escherichia fergusonii*, contributing to a better understanding of their antibiotic resistance profile. The findings presented in this thesis will contribute to the development of more targeted and effective treatment strategies against the Gram-negative bacteria.

2.10 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of an antimicrobial (such as an antifungal, antibiotic, or bacteriostatic) is a medication that inhibits the observable growth of a bacterium after overnight incubation. After isolating a pure culture, MICs can be evaluated on plates of

solid growth medium (called agar, as demonstrated in the "Kirby-Bauer Disk Susceptibility Test") or broth dilution procedures (in liquid growth media).

At first, the serial dilution method was used where the concentration of antibiotic was diluted from one test tube to the next. However, this method was lengthy and could not produce accurate results. Therefore, the $C_1V_1 = C_2V_2$ formula method was employed. Brain Heart Infusion (BHI) broth was used as the media. 5 mL of the BHI broth was poured into each test tube and they were autoclaved. Antibiotics were added to the 5 mL BHI tubes in concentrations calculated using the $C_1V_1 = C_2V_2$ formula. The calculated quantity was withdrawn from the BHI tube before adding the same amount of the relevant antibiotic. Then to each test tube, 100 μL of the bacterial suspension (compared with the MacFarland standard of 0.5) was added. The tubes were then incubated in the shaker incubator for 24 hours at 37℃ and 80 rpm. The turbidity of the tubes was observed after 24 hours, and the MIC of the antibiotic for the specific bacteria sample was determined in the tube with the lowest concentration of clear medium. The same method was repeated for different concentrations of Vitamin C solution and the MIC of Vitamin C for each sample was determined. The experiment was carried out for each antibiotic and Vitamin C in multiple phases to determine the accurate and specific MIC of the antibiotics and Vitamin C alone. Using the results of the individual MIC values of the antibiotics and Vitamin C, different combination dosages of the antibiotic and the Vitamin C were prepared and the MIC value of the combination dose was determined. The combination MIC for the antibiotics and Vitamin C with a clear medium was established as the lowest total concentration. Initially, 15 different antibiotics were chosen and were tested using the antibiotic susceptibility tests. From the results, 2 antibiotics against which all the bacterial samples showed resistance were selected for the MIC experiment. These were-Ceftazidime and Ciprofloxacin. To ensure reliable results, the whole experiment was repeated.

2.11 Calculation of Fractional Inhibitory Concentration Index (FICI)

To evaluate the interaction of the two medications in combination, the fractional inhibitory concentration Index (FICI) was utilized. For the experiment, it was used to determine the interaction between the antibiotic and Vitamin C. The effect of the antibiotic and Vitamin C was classified according to the following criteria-

- FICI $\leq$ 0.5, synergistic effects
- 0.5 < FICI $\leq$ 1, additive effects
- 1 < FICI < 4, no interactions
- FICI $\geq$ 4.0, antagonistic effects

The formula of the FIC Index is-

ΣFIC = FICA + FICB = (CA/MICA) + (CB/MICB), where MICA and MICB are the MICs of drugs A and B alone, respectively, and CA and CB are the concentrations of the drugs in combination.

Using previously collected data, these formulas were used to calculate the FIC index for each sample. After that, the efficacy of each sample was determined by comparing the FIC index to the standards.

2.12 Bacterial Genomic DNA Extraction

The bacterial sample was subjected to a series of procedures, including genomic DNA extraction, PCR, gel electrophoresis, and 16s rRNA sequencing. The bacteria were cultured in Nutrient Agar (NA) for 24 hours. From the culture, a loopful of bacteria was mixed with 150 µL of TE buffer in a 2mL microcentrifuge tube and vortexed. Subsequently, the microcentrifuge tubes were placed in a water bath machine set at 95°C for 20 minutes. After

the incubation, the tubes were removed from the water bath machine and centrifuged at 10,000 rpm for 10 minutes. The supernatants, containing the template DNA, were then collected in another 2 mL microcentrifuge tube and stored at -20°C for PCR analysis and further use.

2.13 Polymerase Chain Reaction (PCR)

The PCR process was conducted with a total volume of 25µl for each sample. The components included 5µl of template DNA, 2.5 µL of the forward primer, 2.5 µl of the reverse primer, 12.5 µl of PCR master mix, and 2.5 µl of nuclease-free water.

The target sequence for PCR was the bacteria's 16s rRNA gene, and universal primers 27F forward and 1492R reverse were utilized. The PCR cycle consisted of initial denaturation at 95°C for 2 minutes, denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, elongation at 72°C for 2 minutes, and a final cycle at 72°C for 7 minutes. A total of 30 cycles were performed during the PCR process. The resulting PCR products were stored at -20°C until further analysis.

2.14 Gel Electrophoresis

Gel electrophoresis was performed in this experiment to verify the presence of PCR products at the desired location. A 1% agarose gel was prepared by mixing 1g of agarose with 100 mL of TAE buffer. The mixture was heated, thoroughly mixed, and then cooled to a semi-warm temperature. To this gel solution, 5 µl of 0.5 µg/mL EtBr was added and mixed. The casting tray was arranged with combs to accommodate multiple samples, and two combs were placed on the casting tray. The gel solution was poured into the casting tray and allowed to solidify. Once solidified, the combs were gently removed, and the tray was placed into the gel electrophoresis machine. Then, TAE running buffer was added, and 6 µl of 100 bp Ladder was carefully placed in the first well of each row, while 6 µl of the PCR products were loaded into

the other wells. The gel was then run at 110 volts for 40 minutes. After the completion of gel electrophoresis, gel bands were observed using UV light, confirming the presence of PCR products at the intended site, thus the presence of DNA in the samples.

2.15 16s rRNA Sequencing

About 50 µl of the PCR product of the samples that showed bands in the PCR result (RR-3, RR-4, RR-5, RR-6) were sent to Invent Technology LTD for 16s rRNA sequencing.

Chapter 3

Results

The bacterial samples were streaked on Nutrient Agar (NA) plates and isolated to get single colonies. The cultures were grown and sub-cultured on NA plates. Colonies were taken from there to inoculate into T1N1 media to make stock and were also inoculated into physiological saline to make the bacterial suspension that was used in the MIC experiments.

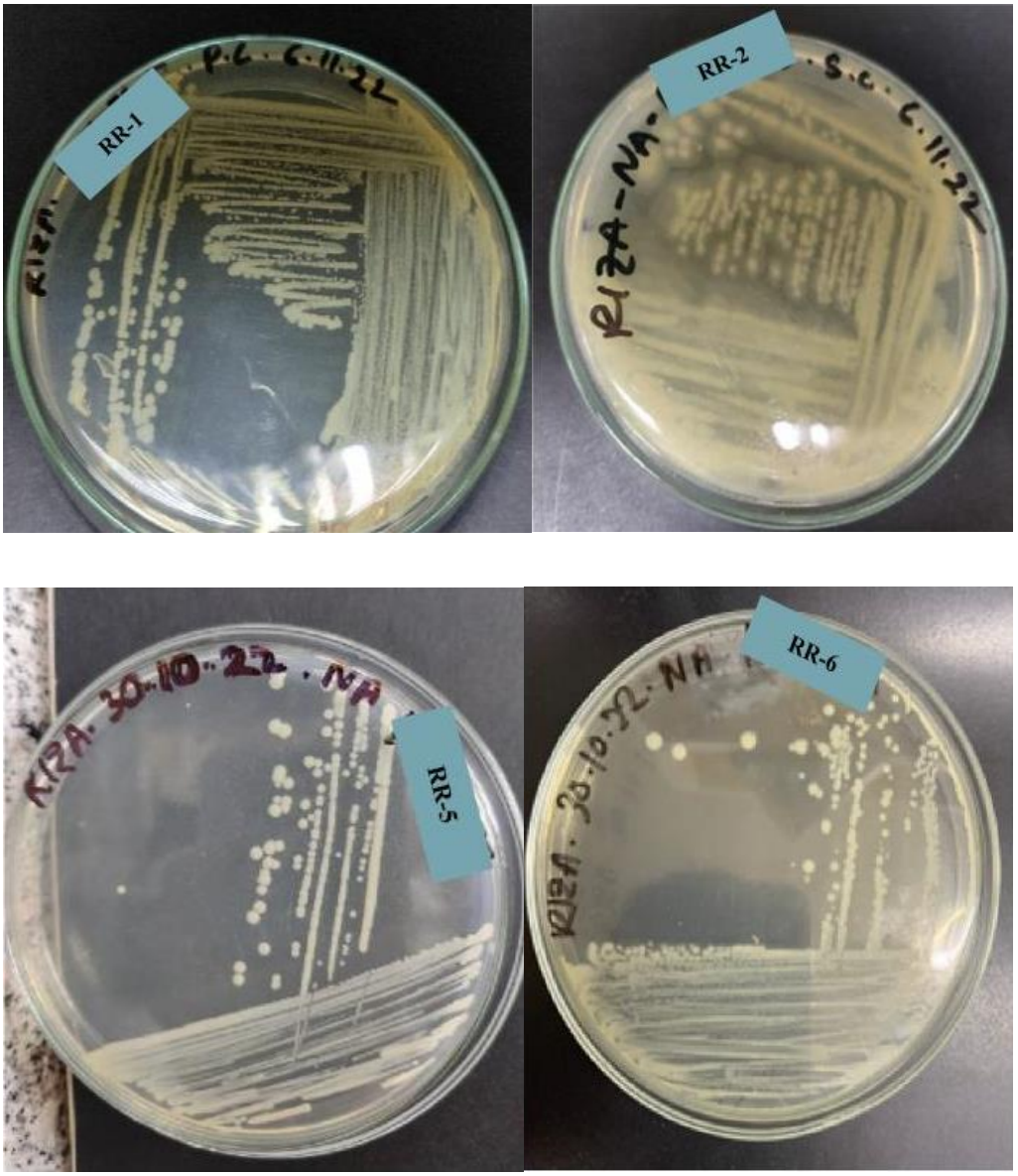


Figure 3: Samples cultured in Nutrient Agar

3.1 Biochemical Tests

3.1.1 Gram Staining

Table 2: Gram Staining Observation

Sample ID	Observation
RR-1	Gram-negative, rod
RR-2	Gram-negative, cocci in clusters
RR-3	Gram-negative, mixed culture
RR-4	Gram-negative, rod
RR-5	Gram-negative cocci
RR-6	Gram-negative, circular

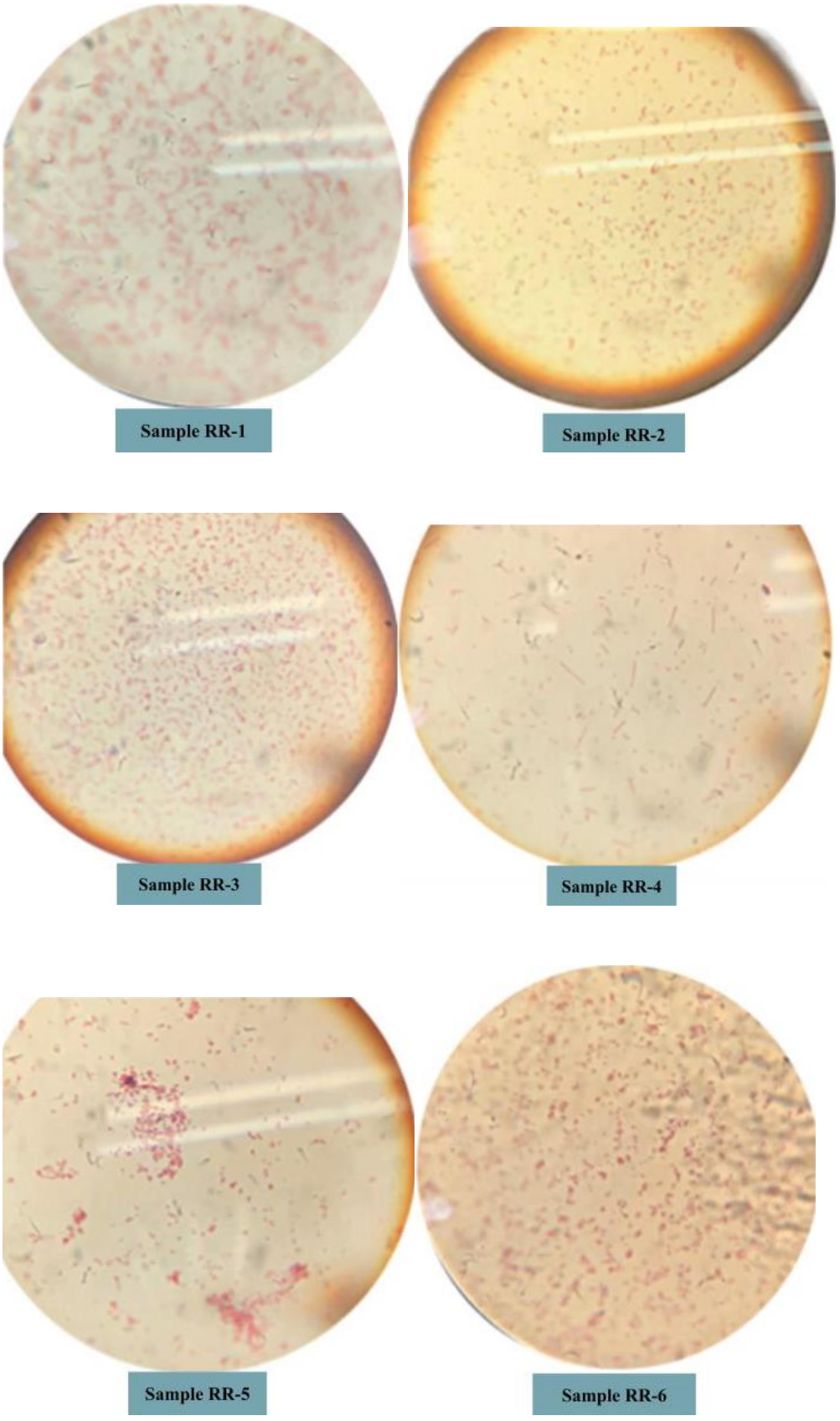


Figure 4: Samples seen under a microscope after Gram-staining.

Table 3: Biochemical Characteristics of Bacterial Samples

Sample ID	Oxidase test	Catalase test	MIU			MRVP		Nitrate reduction	MSA	XLD	Cetrimide agar	TSI						Organism Interpretation
			Motility	Indole	Urease	Methyl Red	Voges Proskauer					Slant/ Butt	Glucose	Lactose	Sucrose	H_2S production	Gas production	
RR-1	-	+	+	+	+	+	+	+	-	R	+	R/Y	+	-	-	-	-	*Pseudomonas sp.*
RR-2	-	+	+	+	+	+	+	+	-	R	+	R/R	-	-	-	-	-	*Pseudomonas sp.*
RR-3	+	+	+	+	+	-	+	-	-	R	+	R/B	+	-	-	+	+	*Pseudomonas sp.*
RR-4	-	+	+	+	+	-	-	+	+	Y	-	Y/Y	+	+	+	-	+	*Escherichia sp.*
RR-5	+	+	+	+	+	+	+	-	-	R	+	R/B	+	-	-	+	+	*Pseudomonas sp.*
RR-6	-	+	+	+	-	+	+	+	-	Y	-	R/B	+	-	-	+	+	*Enterobacter sp.*

R: Red colonies, Y: Yellow colonies, From TSI, B: Black, Y: Yellow, R: Red

3.2 Categorizing Pathogenic Bacteria

A total of six samples were collected which were then tested for an antibiogram. According to the results of the antibiogram, all six samples were multi-drug-resistant (MDR). Four different samples were belonging to the *Pseudomonas* genus: RR-1, RR-2, RR-3, and RR-5. Additionally, there was one sample from the *Escherichia* genus, identified as RR-4, and another sample from the *Enterobacter g*enus, RR-6.

Table 4: Samples Collected

	Pseudomonas	***Escherichia***	***Enterobacter***
Sample ID	RR-1 RR-2 RR-3 RR-5	RR-4	RR-6

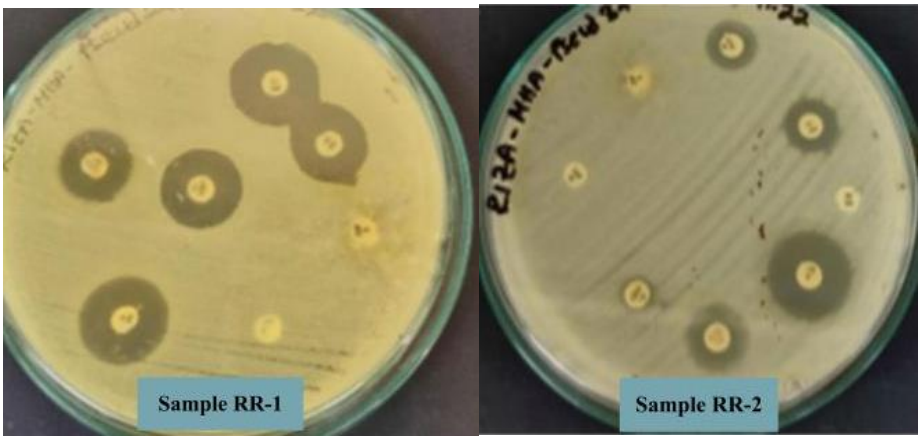
Table 5: Antibiotic Susceptibility Tests

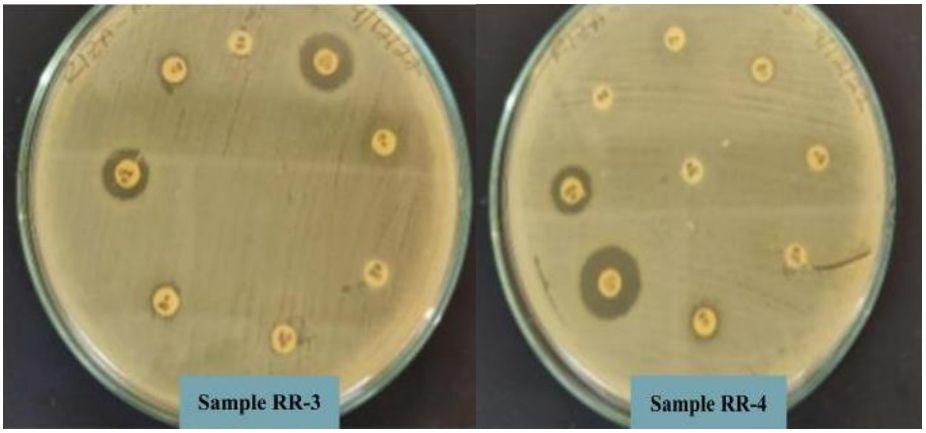
No	**Antibiotic Name**	**Sample ID**					
		RR-1	**RR-2**	**RR-3**	**RR-4**	**RR-5**	**RR-6**
1	Amikacin	S	R	R	R	R	R
2	Ampicillin	S	S	R	R	S	R
3	Azithromycin	R	S	R	R	R	R
4	Cefepime	R	S	S	S	R	R
5	Ceftazidime	R	R	R	R	R	R
6	Ceftriaxone	S	S	S	S	R	R
7	Ciprofloxacin	R	R	R	R	R	R

8	Erythromycin	R	R	R	R	R	R
9	Gentamicin	R	R	R	R	R	R
10	Imipenem	R	S	R	R	R	R
11	Levofloxacin	S	S	R	R	R	S
12	Moxifloxacin	S	S	S	R	R	S
13	Norfloxacin	S	S	S	S	R	S
14	Penicillin	R	R	R	R	R	R
15	Tetracycline	R	R	S	R	S	S

S: Sensitive, R: Resistant

Table 5 shows that all the samples are MDR as they exhibit resistance to more than two antibiotics tested. The highlighted rows indicate the antibiotics to which all the samples were resistant.





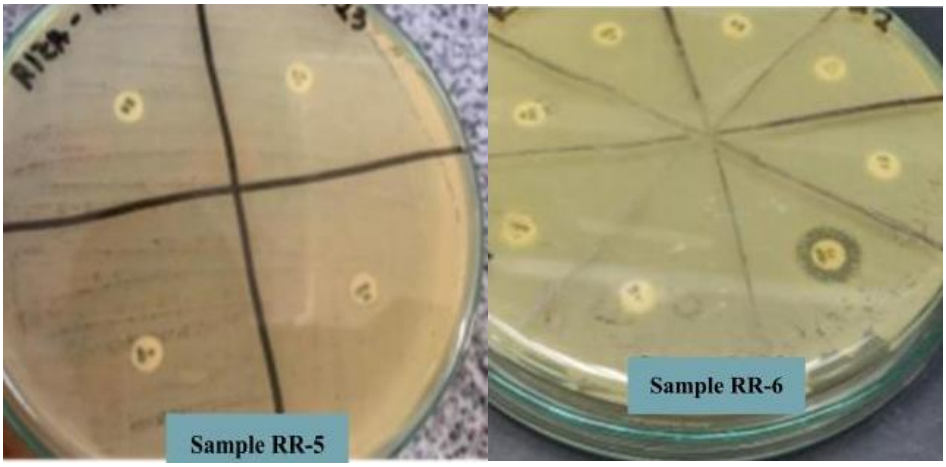


Figure 5: Antibiotic Susceptibility Test

3.3 Determination of Minimum Inhibitory Concentration (MIC)

The effects of Vitamin C on the minimum inhibitory concentrations (MIC) of Ceftazidime and Ciprofloxacin were explored further. The FIC index is utilized as a statistical validation tool when determining synergistic effects. To compare the fractional inhibitory concentration (FIC) to the FIC index, the MIC and FIC were calculated.

3.3.1 Determination of MIC Ceftazidime (CAZ), Vitamin C, and the combination of Ceftazidime and Vitamin C

The provided data from below onwards represents the Minimum Inhibitory Concentration (MIC) values of the antibiotic Ceftazidime, Vitamin C, and the combination MIC of Ceftazidime and Vitamin C for different bacterial strains. The MIC value indicates the lowest concentration of an antibiotic required to inhibit the growth of the bacteria being tested. The creation of these essential concentrations involved multiple repetitions of the $C_1V_1 = C_2V_2$ procedure. Concentrations were measured in micrograms per milliliter (µg/mL), and the bacterial samples were categorized as RR-1, RR-2, RR-3, RR-4, RR-5, and RR-6. The different testing phases provide insights into the impact of the antibiotic, Vitamin C, and their combination on inhibiting bacterial growth.

Table 6: MIC Values of Ceftazidime on the collected samples

Phases	Sample ID					
	RR-1	RR-2	RR-3	RR-4	RR-5	RR-6
	MIC in µg/mL					
Phase 1	100	80	500	300	300	300
Phase 2	60	80	460	300	350	270
Average	80	80	480	300	325	285

Table 6. shows that, in the initial testing phase, a range of MIC values was observed, showcasing the adaptability of ceftazidime. RR-1 exhibited an MIC of 100 µg/mL, while RR-2 showed 80 µg/mL. Although RR-3 posed a bit of a challenge with an MIC of 500 µg/mL, RR-4, RR-5, and RR-6 showed consistent sensitivity with an MIC of 300 µg/mL. Moving on to the second testing phase, it was noted that some shifts in MIC values. RR-1 displayed even greater susceptibility with a MIC value of 60 µg/mL, reinforcing its potential as a candidate for ceftazidime treatment. RR-2 maintained its 80 µg/mL MIC, suggesting a stable response. While RR-3 presented a lower MIC of 460 µg/mL, RR-4, RR-5, and RR-6 exhibited varying degrees of sensitivity, highlighting the adaptability of ceftazidime.

The average MIC values show that ceftazidime consistently performs well across the board. RR-1 and RR-2 showed sensitivity, remaining stable at 80 µg/mL. However, RR-3 showed a different MIC value of 480 µg/mL, suggesting a specific response profile. For RR-4, RR-5, and RR-6, the story remains consistent. Their average MIC values stayed around 300 µg/mL, 325 µg/mL, and 285 µg/mL, respectively. This consistent pattern of sensitivity underlines the reliability of ceftazidime in inhibiting bacterial growth across these diverse samples.

Table 7: MIC Values of Vitamin C on collected samples

Phases	Sample ID					
	RR-1	RR-2	RR-3	RR-4	RR-5	RR-6
	MIC in µg/mL					
Phase 1	200	200	100	200	300	1100
Phase 2	135	165	100	200	295	1100
Average	167.5	182.5	100	200	297.5	1100

Table 7 illustrates that, during the first testing phase, a range of MIC values was discovered, signifying the adaptability of Vitamin C. RR-1, RR-2, and RR-4 consistently demonstrated sensitivity with a MIC of 200 µg/mL, while RR-3 showcased a MIC of 100 µg/mL. In contrast, RR-4 and RR-5 presented MIC values of 300 and 1100 µg/mL, respectively. In the second phase, Vitamin C's effectiveness showed promising improvements compared to the initial phase. The MIC values for all samples in the second phase were generally lower, indicating better sensitivity to Vitamin C.

During the second testing phase, the MIC values continued to reveal Vitamin C's adaptability. RR-1 had an MIC of 135 µg/mL, while RR-2 showed 165 µg/mL. RR-3 exhibited 100 µg/mL, RR-4 had 200 µg/mL, RR-5 showed 295 µg/mL, and RR-6 had an MIC of 1100 µg/mL. This suggests that Vitamin C may be more effective in inhibiting bacterial growth during the second phase of testing. These values, when averaged, presented an intriguing picture. The average MIC values for RR-1 and RR-2 were 167.5 µg/mL and 182.5 µg/mL, respectively. RR-3 maintained a consistently low MIC value of 100 µg/mL, emphasizing its responsiveness to Vitamin C. RR-4 and RR-5 demonstrated moderate MIC values of 200 µg/mL and 297.5

μg/mL, respectively, suggesting a balanced sensitivity. In contrast, RR-6 exhibited the highest MIC value, indicating its relative resistance to Vitamin C at 1100 μg/mL

Table 8: MIC Values of Combination dosages of Ceftazidime and Vitamin C

Sample ID	MIC in μg/mL	
	Ceftazidime	**Vitamin C**
RR-1	**70**	**100**
RR-2	**150**	**150**
RR-3	**200**	**200**
RR-4	**260**	**160**
RR-5	**300**	**300**
RR-6	**300**	**500**

Table 8 shows the combination testing phase. The MIC values for ceftazidime and Vitamin C were assessed. RR-1 displayed a response with MIC values of 70 μg/mL for ceftazidime and 100 μg/mL for Vitamin C. However, RR-2 showed MIC at a combination of 150 μg/mL for ceftazidime and Vitamin C each. RR-5 and RR-6 showed MIC at very high concentrations of ceftazidime and Vitamin C, where for RR-5, it was 300 μg/mL for both CAZ and Vitamin C, and RR-6 showed 300 μg/mL of CAZ and 500 μg/mL for Vitamin C. RR-3 demonstrated an MIC of 200 μg/mL for both Vitamin C and ceftazidime.

Table 9: Determination of Arithmetic Mean MIC value of Ceftazidime, Vitamin C and their combination

Sample ID	MIC (in µg/mL)				FIC Index*
	Ceftazidime only	Vitamin-C only	Ceftazidime + Vitamin-C		
			CAZ	Vit-C	
RR-1	80	167.5	70	100	3.14
RR-2	80	182.5	150	150	5.40
RR-3	480	100	200	200	4.83
RR-4	300	298	167.5	117.5	1.91
RR-5	275	297.5	300	300	4.20
RR-6	285	1066	300	500	3.60

3.3.2 Determination of MIC of Ciprofloxacin, Vitamin C, and the combination of Ciprofloxacin and Vitamin C

The presented data from below onwards provides Minimum Inhibitory Concentration (MIC) values for Ciprofloxacin, Vitamin C, and combinations of Ciprofloxacin and Vitamin C, specifically targeting various distinct Gram-negative bacteria, including those from the *Pseudomonas* genus, *Escherichia* genus, and *Enterobacter* genus. These MIC values are measured in micrograms per milliliter (µg/mL). The different phases of testing yield insights into how the antibiotic, Vitamin C, and their combination affect the inhibition of bacterial growth in these specific Gram-negative bacterial groups.

Table 10: MIC Values of Ciprofloxacin Phase 1

Antibiotic Name	Antibiotic concentration (µg/mL)	Samples					
Ciprofloxacin		RR-1	RR-2	RR-3	RR-4	RR-5	RR-6
	60	T	T	T	C	T	T
	70	T	T	T	C	T	T
	80	T	C	C	C	T	T
	100	T	C	C	C	T	T
	200	C	C	C	C	C	T
	300	C	C	C	C	C	C

T: Turbid, C: Clear, C: MIC

Table 10. shows the initial phase of testing Ciprofloxacin, here MIC values were observed to be 200 µg/mL for RR-1, 80 µg/mL for RR-2, 80 µg/mL for RR-3, 60 µg/mL for RR-4, 200 µg/mL for RR-5, and 300 µg/mL for RR-6. These results indicate that RR-4 was the most susceptible to Ciprofloxacin, requiring the lowest concentration to inhibit growth. RR-5 and RR-6 generally exhibited higher MIC values, suggesting reduced sensitivity to the antibiotic compared to RR-1, RR-2, RR-3, and RR-4.

Table 11: MIC Values of Vitamin C Phase 1

Vitamin name	Antibiotic concentration (µg/mL)	Sample ID					
		RR-1	RR-2	RR-3	RR-4	RR-5	RR-6
Ascorbic Acid	60	T	T	T	T	T	T
	70	T	T	T	T	T	T
	80	T	T	T	T	T	T
	100	T	T	C	T	T	T
	200	C	C	C	C	T	T
	300	C	C	C	C	C	T
	400	C	C	C	C	C	T
	500	C	C	C	C	C	T
	600	C	C	C	C	C	T
	700	C	C	C	C	C	T
	800	C	C	C	C	C	T
	900	C	C	C	C	C	T
	1000	C	C	C	C	C	T
	1100	C	C	C	C	C	C

T: Turbid, C: Clear, C: MIC

Table 12 shows the initial stage of Vitamin C testing, here the MIC values were 200 µg/mL for RR-1, 200 µg/mL for RR-2, 100 µg/mL for RR-3, 200 µg/mL for RR-4, 300 µg/mL for RR-5, and 1100 µg/mL for RR-6. Overall, these results suggest varying sensitivities of the bacterial strains to Vitamin C, with RR-5 and RR-6 generally requiring higher concentrations to inhibit growth compared to RR-1, RR-2, RR-3, and RR-4.

Table 12: MIC Values of Ciprofloxacin Phase 2

Antibiotic Name	Antibiotic concentration (µg/mL)	Sample ID					
		RR-1	RR-2	RR-3	RR-4	RR-5	RR-6
Ciprofloxacin	40	T	T	T	T	T	T
	45	T	T	T	T	T	T
	50	T	T	C	T	T	T
	60	T	T	C	T	T	T
	65	T	T	C	T	T	T
	70	T	T	C	T	T	T
	75	T	T	C	C	T	T
	80	T	C	C	C	T	T
	85	T	C	C	C	T	T
	90	T	C	C	C	T	T
	100	T	C	C	C	T	T
	105	T	C	C	C	T	T
	110	C	C	C	C	T	T
	115	C	C	C	C	T	T
	120	C	C	C	C	T	T

Ciprofloxacin	**130**	**C**	**C**	**C**	**C**	**T**	**T**
	135	**C**	**C**	**C**	**C**	**T**	**T**
	140	**C**	**C**	**C**	**C**	**T**	**T**
	145	**C**	**C**	**C**	**C**	**T**	**T**
	150	**C**	**C**	**C**	**C**	**T**	**T**
	160	**C**	**C**	**C**	**C**	**T**	**T**
	170	**C**	**C**	**C**	**C**	**T**	**T**
	175	**C**	**C**	**C**	**C**	**T**	**T**
	180	**C**	**C**	**C**	**C**	**C**	**T**
	185	**C**	**C**	**C**	**C**	**C**	**T**
	190	**C**	**C**	**C**	**C**	**C**	**T**
	195	**C**	**C**	**C**	**C**	**C**	**T**
	200	**C**	**C**	**C**	**C**	**C**	**T**
	260	**C**	**C**	**C**	**C**	**C**	**T**
	270	**C**	**C**	**C**	**C**	**C**	**C**

T: Turbid , C: Clear, C: MIC

Table 12. showed that during the second testing phase, the MIC values for Ciprofloxacin were determined. Here, the MIC values were 110 µg/mL for RR-1, 80 µg/mL for RR-2, 50 µg/mL for RR-3, 75 µg/mL for RR-4, 180 µg/mL for RR-5, and 270 µg/mL for RR-6. Comparing the

antibiotic Ciprofloxacin's MIC values between the first and second testing phases reveals a notable shift in bacterial sensitivity. In the initial phase, higher concentrations were required for inhibition, while the second phase showed decreased MIC values across strains. This suggests increased bacterial susceptibility to Ciprofloxacin in the later phase.

Table 13: MIC Values of Vitamin C Phase 2

Vitamin name	Concentration of Vitamin C in µg/mL	Sample ID					
		RR-1	RR-2	RR-3	RR-4	RR-5	RR-6
Ascorbic Acid	60	T	T	T	T	T	T
	70	T	T	T	T	T	T
	75	T	T	C	T	T	T
	80	T	T	C	T	T	T
	85	T	T	C	T	T	T
	90	T	T	C	T	T	T
	95	T	T	C	T	T	T
	100	T	T	C	T	T	T
	105	T	T	C	T	T	T
	110	T	T	C	T	T	T
	115	T	T	C	T	T	T
	120	T	T	C	T	T	T
	125	T	T	C	T	T	T
	130	T	T	C	T	T	T
	135	C	T	C	T	T	T
	140	C	T	C	T	T	T

Ascorbic Acid	**145**	**C**	**T**	**C**	**T**	**T**	**T**
	150	**C**	**T**	**C**	**T**	**T**	**T**
	155	**C**	**T**	**C**	**T**	**T**	**T**
	160	**C**	**T**	**C**	**T**	**T**	**T**
	165	**C**	**C**	**C**	**T**	**T**	**T**
	170	**C**	**C**	**C**	**T**	**T**	**T**
	175	**C**	**C**	**C**	**T**	**T**	**T**
	180	**C**	**C**	**C**	**T**	**T**	**T**
	185	**C**	**C**	**C**	**T**	**T**	**T**
	190	**C**	**C**	**C**	**T**	**T**	**T**
	195	**C**	**C**	**C**	**T**	**T**	**T**
	200	**C**	**C**	**C**	**C**	**T**	**T**
	270	**C**	**C**	**C**	**C**	**T**	**T**
	280	**C**	**C**	**C**	**C**	**T**	**T**
	285	**C**	**C**	**C**	**C**	**T**	**T**
	290	**C**	**C**	**C**	**C**	**T**	**T**
	295	**C**	**C**	**C**	**C**	**C**	**T**
	700	**C**	**C**	**C**	**C**	**C**	**T**
	800	**C**	**C**	**C**	**C**	**C**	**T**
	900	**C**	**C**	**C**	**C**	**C**	**T**
	1000	**C**	**C**	**C**	**C**	**C**	**C**
	1100	**C**	**C**	**C**	**C**	**C**	**C**

T: Turbid , C: Clear, C: MIC

Table 13 shows the second testing phase, here the MIC values for Vitamin C were determined. Here, the MIC values were 135 μg/mL for RR-1, 165 μg/mL for RR-2, 75 μg/mL for RR-3, 200 μg/mL for RR-4, 295 μg/mL for RR-5, and 1100 μg/mL for RR-6. Notably, RR-6 exhibited a higher MIC value, suggesting a greater resistance to Vitamin C compared to other strains. Comparing the two phases of Vitamin C testing, the initial stage showed higher MIC values, indicating greater resistance among RR-5 and RR-6. RR-1, RR-2, RR-3, and RR-4 demonstrated moderate sensitivity. In the second phase, all the strains displayed decreased MIC values, suggesting improved sensitivity to Vitamin C, especially noticeable in RR-1 to RR-4.

Table 14: MIC Values of Ciprofloxacin and Vitamin C combination

Concentrations of Antibiotic and Vitamin C in µg/mL		Sample ID					
Ciprofloxacin	**Vitamin C**	**RR-1**	**RR-2**	**RR-3**	**RR-4**	**RR-5**	**RR-6**
10	15	C	–	–	C	–	–
10	20	–	–	–	C	–	–
15	5	–	–	T	–	–	–
15	10	C	–	–	–	–	–
15	15	–	T	–	–	–	–
15	25	–	–	–	C	T	T
15	30	–	–	–	C	–	–
20	20	C	–	–	–	–	–
20	5	–	–	–	C	–	–
20	25	–	–	–	–	T	T
25	5	–	T	–	–	–	–
25	10	–	T	T	–	–	–
25	20	–	–	–	–	–	–
25	25	C	–	–	–	C	T
25	35	–	–	–	–	C	C

30	**30**	–	–	**C**	–	–	**T**
30	**20**	–	–	–	–	**T**	–
35	**25**	–	**C**	–	–	–	–
35	**10**	–	–	**T**	–	–	–

T: Turbid, C: Clear, C:MIC, --: MIC not carried out in the combination

Table 14. shows the the combination phase, here Minimum Inhibitory Concentration (MIC) values were evaluated for various bacterial strains subjected to combinations of antibiotics and Vitamin C. Specifically, for RR-1, MIC values of (10 µg/mL,15 µg/mL) and (15 µg/mL,10 µg/mL) were observed, while RR-2 exhibited a MIC value of (35 µg/mL,25 µg/mL). For RR-4, a range of MIC values was observed, including (10 µg/mL,15 µg/mL), (10 µg/mL,20 µg/mL), (15 µg/mL,25 µg/mL), (15 µg/mL,30 µg/mL), (20 µg/mL,5 µg/mL), and (35 µg/mL,25 µg/mL). RR-3 displayed a MIC value of (30 µg/mL,30 µg/mL), while RR-6 had a MIC value of (25 µg/mL,35 µg/mL). Notably, RR-5 displayed MIC values of (25 µg/mL,35 µg/mL) and (25 µg/mL,25 µg/mL).

Table 15: Repeat MIC values of Ciprofloxacin Phase 1

The MIC experiments were repeated to get reliable results.

Antibiotic Name	Antibiotic concentration (µg/mL)	Samples					
		RR-1	RR-2	RR-3	RR-4	RR-5	RR-6
Ciprofloxacin	20	T	T	C	T	T	T
	30	T	T	C	T	T	T
	40	T	T	C	C	T	T
	45	T	T	C	C	T	T
	50	T	T	C	C	T	T
	55	T	T	C	C	T	T
	60	T	C	C	C	T	T
	70	T	C	C	C	T	T
	75	T	C	C	C	T	T
	80	T	C	C	C	T	T
	85	T	C	C	C	T	T
	90	T	C	C	C	T	T
	95	T	C	C	C	T	T
	100	T	C	C	C	T	T
	110	T	C	C	C	T	T

Ciprofloxacin	**120**	**T**	**C**	**C**	**C**	**T**	**T**
	130	**T**	**C**	**C**	**C**	**T**	**T**
	140	**C**	**C**	**C**	**C**	**T**	**T**
	150	**C**	**C**	**C**	**C**	**T**	**T**
	160	**C**	**C**	**C**	**C**	**T**	**T**
	165	**C**	**C**	**C**	**C**	**T**	**T**
	170	**C**	**C**	**C**	**C**	**T**	**C**
	180	**C**	**C**	**C**	**C**	**T**	**C**
	190	**C**	**C**	**C**	**C**	**T**	**C**
	195	**C**	**C**	**C**	**C**	**T**	**C**
	200	**C**	**C**	**C**	**C**	**C**	**C**

T: Turbid, C: Clear, C:MIC

Table 15. illustrates, the repeated first phase of Ciprofloxacin testing, the MIC values were observed: 140 µg/mL for RR-1, 60 µg/mL for RR-2, 20 µg/mL for RR-3, 40 µg/mL for RR-4, 200 µg/mL for RR-5, and 170 µg/mL for RR-6. These results indicated that lower Ciprofloxacin concentrations were effective in inhibiting bacterial growth, while higher concentrations demonstrated resistance.

Table 16: Repeat MIC value of Ciprofloxacin Phase 2

Antibiotic Name	Antibiotic conc (µg/mL)	Sample ID					
Ciprofloxacin		RR-1	RR-2	RR-3	RR-4	RR-5	RR-6
	5	T	T	T	T	T	T
	10	T	T	T	T	T	T
	15	T	T	T	T	T	T
	20	T	T	C	T	T	T
	25	T	T	C	T	T	T
	30	T	T	C	T	T	T
	35	T	T	C	T	T	T
	40	T	T	C	C	T	T
	45	T	T	C	C	T	T

	50	**T**	**T**	**C**	**C**	**T**	**T**
	55	**T**	**T**	**C**	**C**	**T**	**T**
	60	**T**	**C**	**C**	**C**	**T**	**T**
	70	**T**	**C**	**C**	**C**	**T**	**T**
	75	**T**	**C**	**C**	**C**	**T**	**T**
	80	**T**	**C**	**C**	**C**	**T**	**T**
	85	**T**	**C**	**C**	**C**	**T**	**T**
	90	**T**	**C**	**C**	**C**	**T**	**T**
	95	**C**	**C**	**C**	**C**	**T**	**T**
	100	**C**	**C**	**C**	**C**	**T**	**T**
	110	**C**	**C**	**C**	**C**	**T**	**T**
	120	**C**	**C**	**C**	**C**	**T**	**T**
	130	**C**	**C**	**C**	**C**	**T**	**T**
	140	**C**	**C**	**C**	**C**	**T**	**T**
	150	**C**	**C**	**C**	**C**	**T**	**T**
	160	**C**	**C**	**C**	**C**	**T**	**T**
	165	**C**	**C**	**C**	**C**	**T**	**T**
	170	**C**	**C**	**C**	**C**	**T**	**C**
	180	**C**	**C**	**C**	**C**	**T**	**C**
	185	**C**	**C**	**C**	**C**	**T**	**C**
	190	**C**	**C**	**C**	**C**	**T**	**C**
	195	**C**	**C**	**C**	**C**	**T**	**C**
	200	**C**	**C**	**C**	**C**	**C**	**C**
	205	**C**	**C**	**C**	**C**	**C**	**C**

T: Turbid, C: Clear, C: MIC

Table 16. shows the repeated second phase of Ciprofloxacin testing, the MIC values remained almost similar for most strains, (except for RR-1) signifying that the strains' responses to the

antibiotic treatment were reproducible. This suggests that the observed trends in bacterial sensitivities were consistent across multiple test repetitions.

Table 17: Repeat MIC value of Vitamin C

Vitamin Name	Antibiotic concentration (µg/mL)	Samples					
		RR-1	RR-2	RR-3	RR-4	RR-5	RR-6
Ascorbic Acid	60	T	T	T	T	T	T
	70	T	T	T	T	T	T
	75	T	T	T	T	T	T
	80	T	T	T	T	T	T
	85	T	T	T	T	T	T
	90	T	T	T	T	T	T
	95	T	T	T	T	T	T
	100	T	T	T	T	T	T
	105	T	T	C	T	T	T
	110	T	T	C	T	T	T
	115	T	T	C	T	T	T
	120	T	T	C	T	T	T
	125	T	T	C	T	T	T
	130	T	T	C	T	T	T

135	**T**	**T**	**C**	**T**	**T**	**T**
140	**T**	**C**	**C**	**T**	**T**	**T**
145	**T**	**C**	**C**	**T**	**T**	**T**
150	**T**	**C**	**C**	**C**	**T**	**T**
160	**T**	**C**	**C**	**C**	**T**	**T**
165	**T**	**C**	**C**	**C**	**T**	**T**
170	**T**	**C**	**C**	**C**	**T**	**T**
180	**C**	**C**	**C**	**C**	**T**	**T**
190	**C**	**C**	**C**	**C**	**T**	**T**
195	**C**	**C**	**C**	**C**	**T**	**T**
200	**C**	**C**	**C**	**C**	**T**	**T**
205	**C**	**C**	**C**	**C**	**T**	**T**
210	**C**	**C**	**C**	**C**	**T**	**T**
220	**C**	**C**	**C**	**C**	**T**	**T**
230	**C**	**C**	**C**	**C**	**T**	**T**
240	**C**	**C**	**C**	**C**	**C**	**C**

T: Turbid, C: Clear, C: MIC

Table 17 shows the repeated first phase of Vitamin C testing. Here the MIC values were noted as follows: 180 µg/mL for RR-1, 140 µg/mL for RR-2, 105 µg/mL for RR-3, 150 µg/mL for RR-4, 240 µg/mL for RR-5, and 240 µg/mL for RR-6. These findings indicate the

concentrations at which Vitamin C effectively inhibited the growth of the respective bacterial strains.

Table 18: MIC Value of Ciprofloxacin and Vitamin C-repeated

Concentration of Antibiotic and Vitamin C in µg/mL		Sample ID					
Ciprofloxacin	**Vitamin C**	**RR-1**	**RR-2**	**RR-3**	**RR-4**	**RR-5**	**RR-6**
5	5	--	--	--	T	--	--
5	15	--	C	--	--	--	--
10	5	--	T	--	--	--	--
10	10	C	C	--	T	--	--
10	20	C	--	--	--	--	--
10	25	C	--	--	--	--	--
10	30	--	--	--	--	--	C
10	40	--	--	--	--	--	C
10	50	--	--	--	--	--	--
15	5	--	C	T	--	--	--
15	15	C	--	--	C	--	--
20	20	--	--	--	--	--	T
20	30	--	--	--	--	--	C
20	40	--	--	--	--	C	--
20	50	--	--	--	--	C	--

25	**10**	--	--	**T**	--	--	--
25	**25**	--	--	**T**	--	**T**	**C**
30	**30**	--	--	**C**	--	**T**	--
35	**35**	--	--	--	--	**C**	--

T: Turbid, C: Clear, C: MIC, --: MIC not carried out in the combination

Table 18 illustrate the combination phase, the Minimum Inhibitory Concentration (MIC) values were evaluated for various bacterial strains subjected to combinations of antibiotics and Vitamin C. Specifically, for RR-1, MIC values of (10 µg/mL, 20 µg/mL) and (10 µg/mL,25 µg/mL) (10 µg/mL,30 µg/mL) were observed, while RR-2 exhibited a MIC value of (5 µg/mL,15 µg/mL)(15 µg/mL,20 µg/mL)(10 µg/mL,10 µg/mL). RR-3 displayed a MIC value of (30 µg/mL,30 µg/mL), for RR-4, a range of MIC values was observed, including (15 µg/mL,15 µg/mL), while RR-6 had a MIC value of (20 µg/mL,30 µg/mL) (25 µg/mL,25 µg/mL). Notably, RR-5 displayed MIC values of (25 µg/mL,40 µg/mL) and (20 µg/mL,50 µg/mL µg/mL) (35 µg/mL,35). These combinations are done based on repeat results of Ciprofloxacin and Vitamin C phases. Certain combinations influenced pH, which might have affected the outcomes.

3.3.3 Changes in pH

The pH of the BHI media was recorded before the incubation with bacteria and antibiotic and (or) Vitamin C and after the incubation. In all cases, the pH remained the same indicating that bacterial growth was inhibited only due to the action of antibiotic and Vitamin C, not due to the pH change.

3.3.4 Determination of the arithmetic mean MIC value of Ciprofloxacin, Vitamin C, and their combination

Table 19: Arithmetic Mean MIC Value of Ciprofloxacin, Vitamin C, and their Combination

Sample ID	MIC (in µg/mL)				Mean FIC Index*
	Ciprofloxacin only (mean)	Vitamin-C only (mean)	Ciprofloxacin + Vitamin-C (mean)		
			CIP	Vit-C	
RR-1	136.25	173.75	10	17.5	0.36
RR-2	70	220	12.5	15	0.51
RR-3	80	300	15	15	0.47
RR-4	58.75	250	7.5	15	0.48
RR-5	195	268.75	22.5	32.5	0.49
RR-6	227.5	270	17.5	32.5	0.41

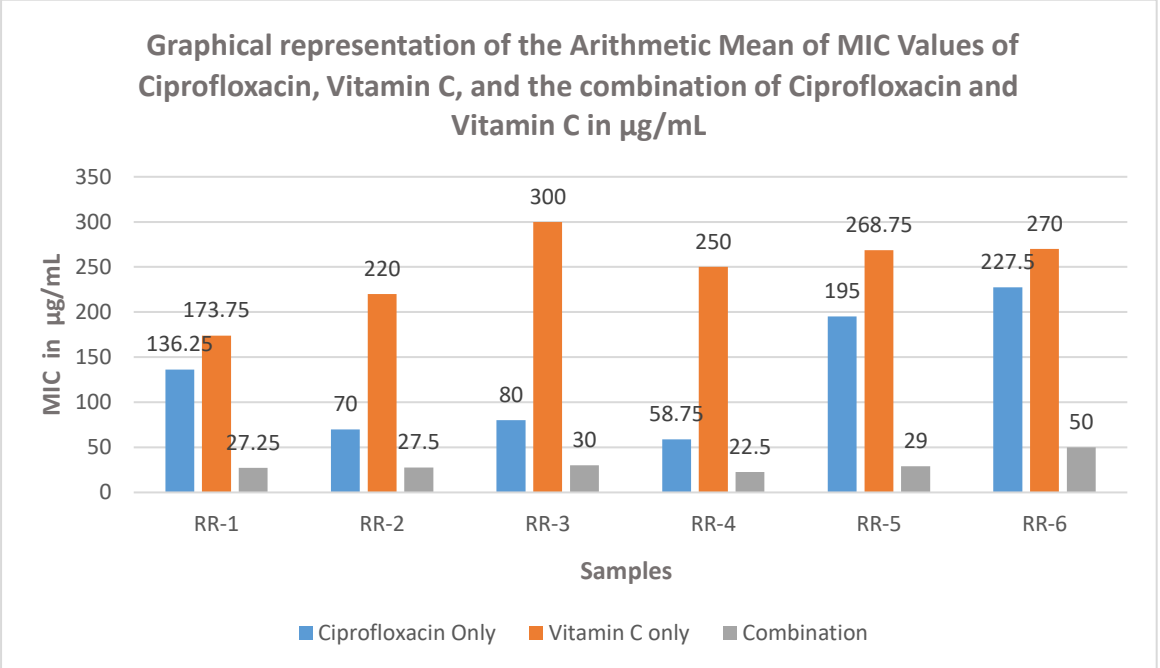


Figure 6: Graphical representation of the Arithmetic Mean of MIC Values of Ciprofloxacin, Vitamin C, and their combination.

The average FICI of Ciprofloxacin and Vitamin C

The arithmetic mean of FICI of the samples are given below-

- **RR-1: 0.36**
- **RR-2: 0.51**
- **RR-3: 0.47**
- **RR-4: 0.48**
- **RR-5: 0.49**
- **RR-6: 0.41**

All the mean FIC indexes were below 0.5 which indicates synergistic effects of Ciprofloxacin and Vitamin C on the strains. For the first phase, the combination MICs were 25 µg/mL (10 µg/mL Ciprofloxacin, 15 µg/mL Vitamin C) for **RR-1**, 30 µg/mL (15 µg/mL Ciprofloxacin, 15 µg/mL Vitamin C) for **RR-4**, 60 µg/mL (30 µg/mL Ciprofloxacin and 30 µg/mL Vitamin C) for **RR-3** and 60 µg/mL (35 µg/mL Ciprofloxacin, 25 µg/mL Vitamin C) for **RR-2**. Later, when the experiment was repeated, the combination MIC of **RR-1** was 30 µg/mL (10,20), for **RR-4** it was 30 µg/mL (15 µg/mL,15 µg/mL), 60 µg/mL for **RR-3** (20 µg/mL,40 µg/mL), (30 µg/mL,30 µg/mL) and 35 µg/mL (15 µg/mL,20) µg/mL for **RR-2**. For the first phase, the combination MICs were 60 µg/mL (25 µg/mL Ciprofloxacin, 35 µg/mL Vitamin C) for **RR-6** and 50 µg/mL (25 µg/mL Ciprofloxacin, 25 µg/mL Vitamin C) for **RR-5**. Later, when the experiment was repeated, the combination MIC of **RR-6** was found at 50 µg/mL (20 µg/mL Ciprofloxacin, 30 µg/mL Vitamin C), and for **RR-5**, it was 70 µg/mL (20 µg/mL Ciprofloxacin and 50 µg/mL Vitamin C). Results of the first run and repeat were added together and mean

MIC Values were calculated and thus the mean FICI were found out. Thus, all the results were close to each other and reliable.

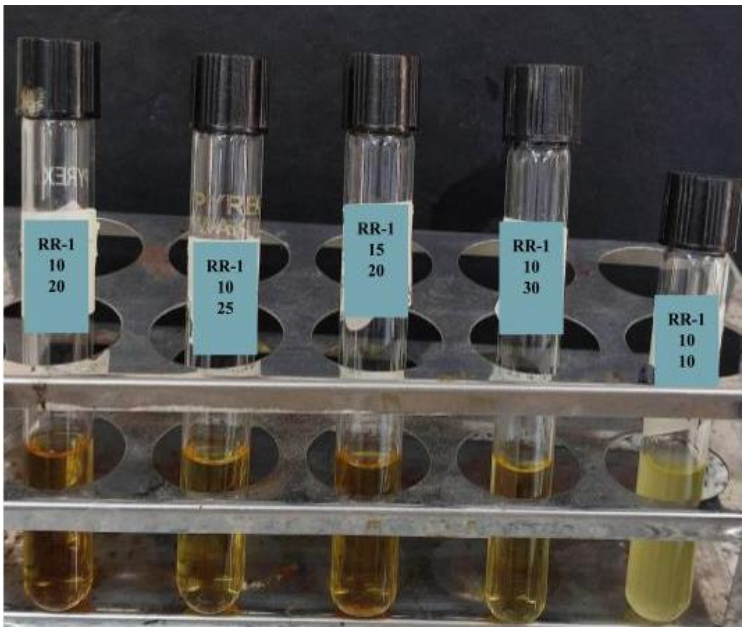


Figure 7: MIC Values of Combination between Ciprofloxacin and Vitamin C for Sample RR-1.

In Figure 7. first number indicates the dose of Ciprofloxacin in µg/mL whereas the second number indicates the dose of Vitamin C in µg/mL. Here the MIC is 30 µg/mL where the concentration of Ciprofloxacin was 10 µg/mL and the concentration of Vitamin C was 20 µg/mL.

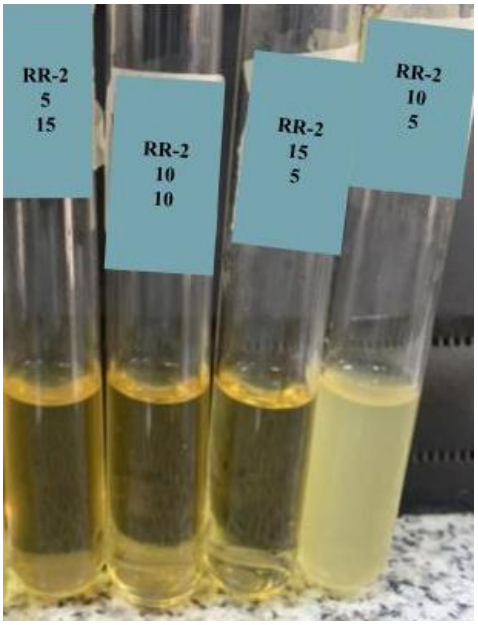


Figure 8: MIC Values of Combination between Ciprofloxacin and Vitamin C for Sample RR-2.

The first number indicates the dose of Ciprofloxacin in µg/mL whereas the second number indicates the dose of Vitamin C in µg/mL. Here the MIC is 20 µg/mL with a range of different combinations like 5 µg/mL Ciprofloxacin, 15 µg/mL Vitamin C, then 10 µg/mL Ciprofloxacin, 10 µg/mL Vitamin C and lastly 15 µg/mL Ciprofloxacin, 5 µg/mL Vitamin C.

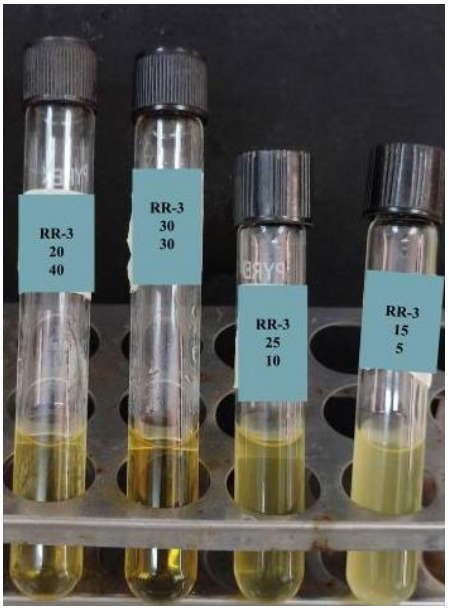


Figure 9: MIC Values of Combination between Ciprofloxacin and Vitamin C for Sample RR-3

The first number indicates the dose of Ciprofloxacin in µg/mL whereas the second number indicates the dose of Vitamin C in µg/mL. Here, the MIC is 35 µg/mL with 25 µg/mL Ciprofloxacin and 10 µg/mL Vitamin C.

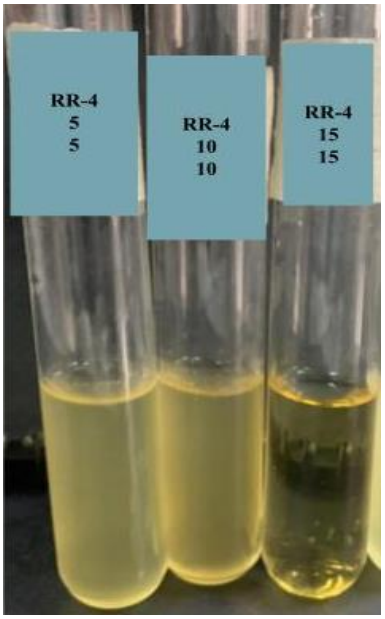


Figure 10: MIC Values of Combination between Ciprofloxacin and Vitamin C for Sample RR-4.

The first number indicates the dose of Ciprofloxacin in µg/mL whereas the second number indicates the dose of Vitamin C in µg/mL. Here, the MIC is 30 µg/mL with 15 µg/mL Ciprofloxacin and 15 µg/mL Vitamin C.

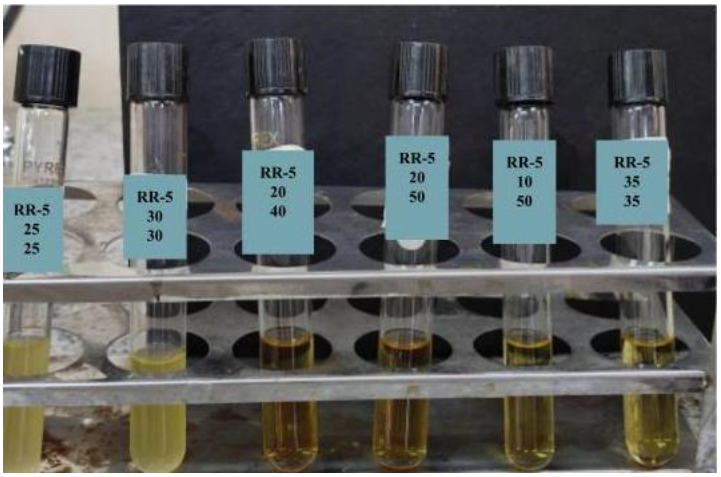


Figure 11: MIC Values of Combination of Ciprofloxacin and Vitamin C for Sample RR-5.

The first number indicates the dose of Ciprofloxacin in µg/mL whereas the second number indicates the dose of Vitamin C in µg/mL. Here, the MIC is 60 µg/mL with two different combinations of 20 µg/mL Ciprofloxacin, 40 µg/mL Vitamin C and 10 µg/mL, 50 µg/mL Vitamin C.

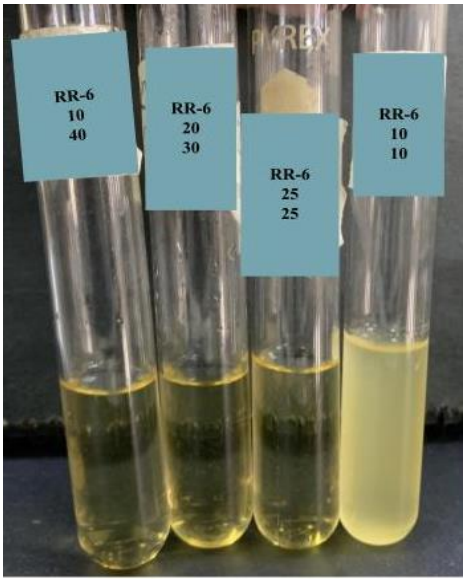


Figure 12: MIC Values of Combination of Ciprofloxacin and Vitamin C for Sample RR-6.

The first number indicates the dose of Ciprofloxacin in µg/mL whereas the second number indicates the dose of Vitamin C in µg/mL. Here, the MIC is 50 µg/mL with three different

combinations of 10 µg/mL Ciprofloxacin, 20 µg/mL Vitamin C and 10 µg/mL, 30 µg/mL Vitamin C, and 25 µg/mL Ciprofloxacin, 25 µg/mL Vitamin C.

3.4 PCR for detecting the presence of DNA in the extracted samples

In the PCR experiment which was run using universal primer, encompassing samples numbered RR-1 through RR-6, it is evident that DNA bands were detected in samples RR-3, RR-4, RR-5, and RR-6. Conversely, samples RR-1, and RR-2 exhibited an absence of discernible DNA bands during the analysis.

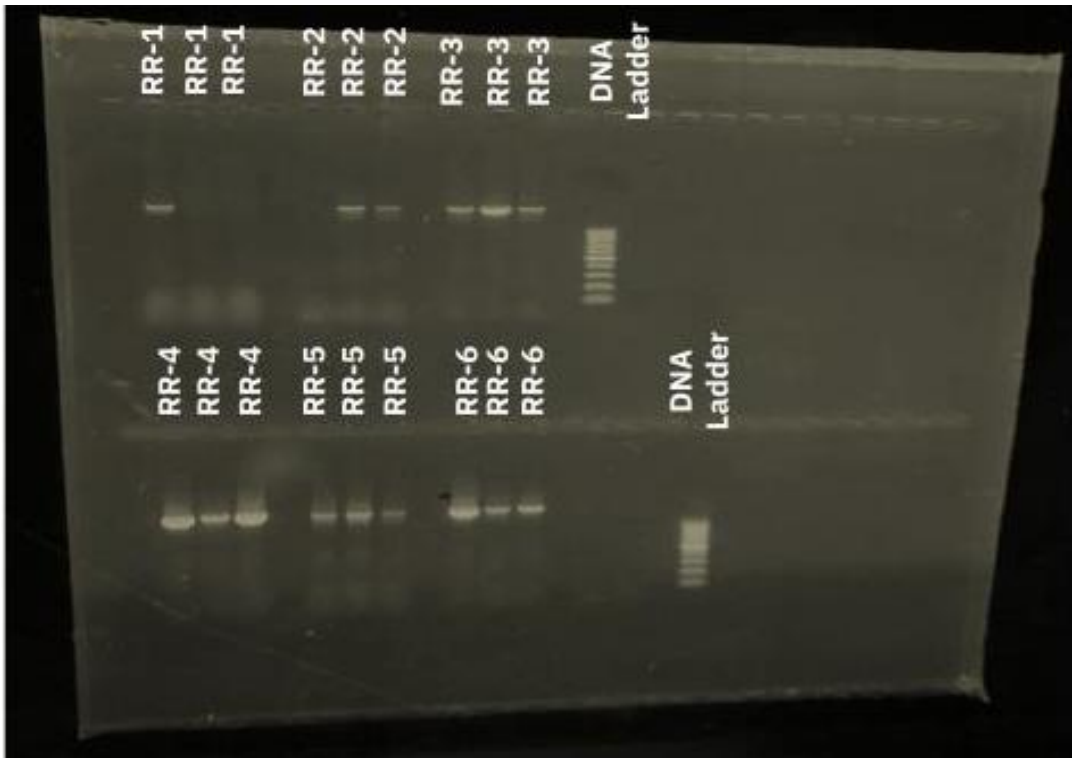
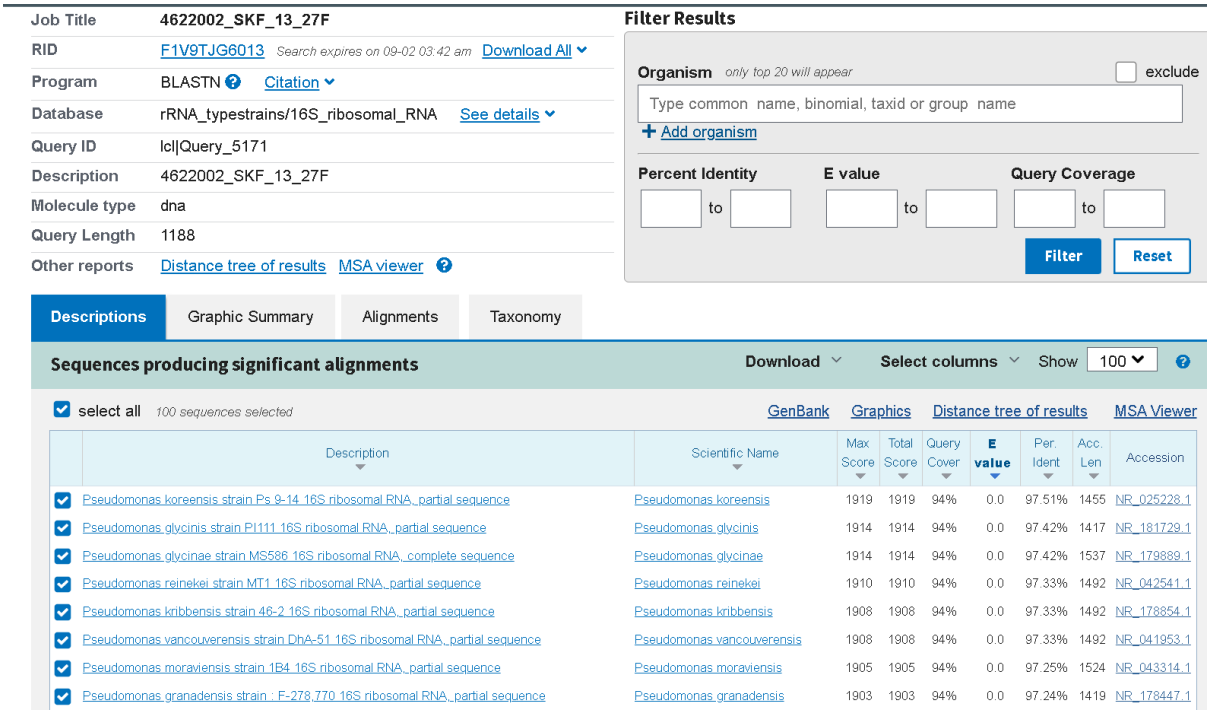


Figure 10: PCR Result with universal primer; it shows bands for RR-3, RR-4, RR-5, and RR-6.

3.5 16s rRNA Sequencing to Identify the Samples

Figure 13: BLAST of Sample RR-3 (Forward Sequence)



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Program	BLASTN Citation
Database	rRNA_typestrains/16S_ribosomal_RNA See details
Query ID	lcl|Query_5171
Description	4622002_SKF_13_27F
Molecule type	dna
Query Length	1188
Other reports	Distance tree of results MSA viewer

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Percent Identity to

E value to

Query Coverage to

Filter Reset

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download Select columns Show 100

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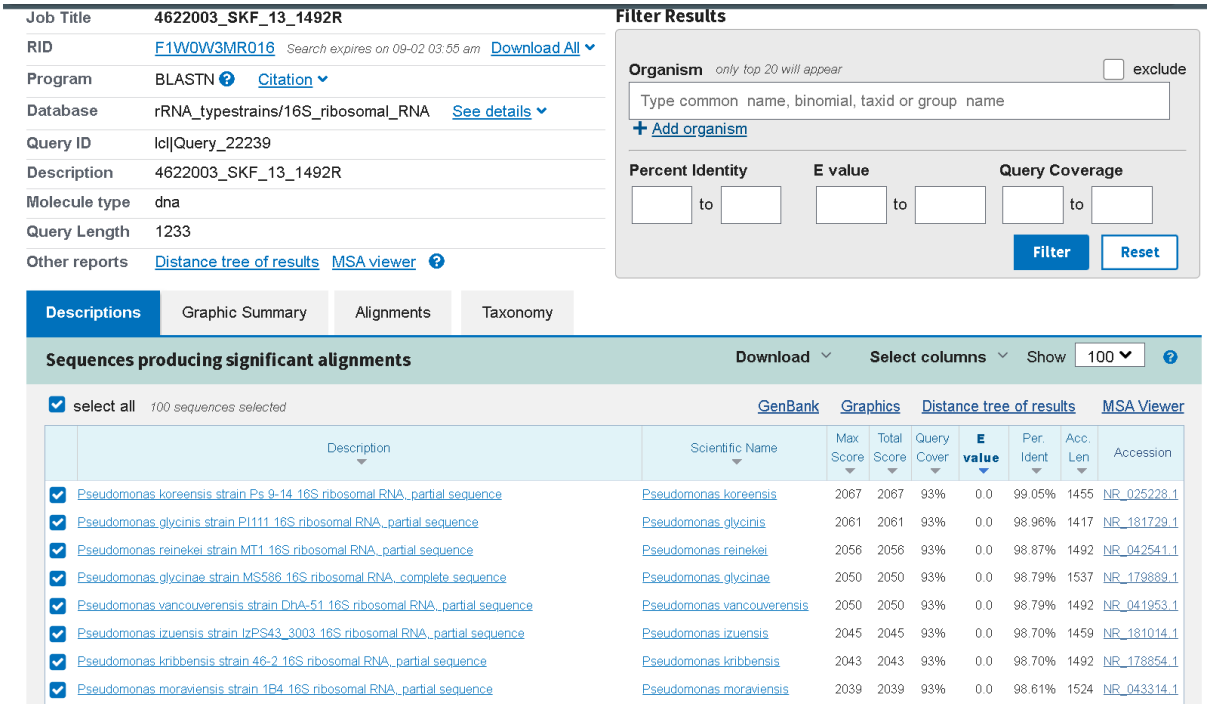
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Pseudomonas koreensis strain Ps 9-14 16S ribosomal RNA, partial sequence	Pseudomonas koreensis	1919	1919	94%	0.0	97.51%	1455	NR_025228.1
Pseudomonas glycinis strain PI111 16S ribosomal RNA, partial sequence	Pseudomonas glycinis	1914	1914	94%	0.0	97.42%	1417	NR_181729.1
Pseudomonas glycinae strain MS586 16S ribosomal RNA, complete sequence	Pseudomonas glycinae	1914	1914	94%	0.0	97.42%	1537	NR_179889.1
Pseudomonas reinekei strain MT1 16S ribosomal RNA, partial sequence	Pseudomonas reinekei	1910	1910	94%	0.0	97.33%	1492	NR_042541.1
Pseudomonas kribbensis strain 46-2 16S ribosomal RNA, partial sequence	Pseudomonas kribbensis	1908	1908	94%	0.0	97.33%	1492	NR_178854.1
Pseudomonas vancouverensis strain DhA-51 16S ribosomal RNA, partial sequence	Pseudomonas vancouverensis	1908	1908	94%	0.0	97.33%	1492	NR_041953.1
Pseudomonas moraviensis strain 1B4 16S ribosomal RNA, partial sequence	Pseudomonas moraviensis	1905	1905	94%	0.0	97.25%	1524	NR_043314.1
Pseudomonas granadensis strain : F-278,770 16S ribosomal RNA, partial sequence	Pseudomonas granadensis	1903	1903	94%	0.0	97.24%	1419	NR_178447.1

Figure 14: BLAST of Sample RR-3 (Reverse Sequence)



Job Title	4622003_SKF_13_1492R
RID	F1W0W3MR016 Search expires on 09-02 03:55 am Download All
Program	BLASTN Citation
Database	rRNA_typestrains/16S_ribosomal_RNA See details
Query ID	lcl|Query_22239
Description	4622003_SKF_13_1492R
Molecule type	dna
Query Length	1233
Other reports	Distance tree of results MSA viewer

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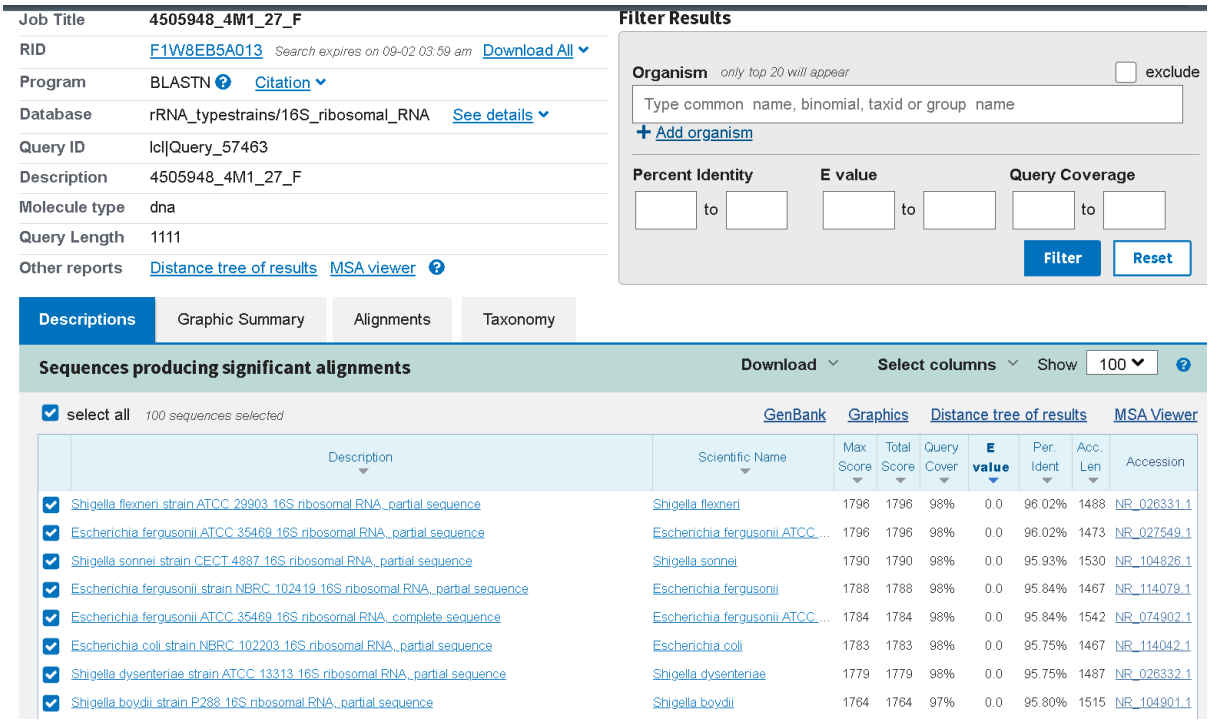
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Pseudomonas koreensis strain Ps 9-14 16S ribosomal RNA, partial sequence	Pseudomonas koreensis	2067	2067	93%	0.0	99.05%	1455	NR_025228.1
Pseudomonas glycinis strain PI111 16S ribosomal RNA, partial sequence	Pseudomonas glycinis	2061	2061	93%	0.0	98.96%	1417	NR_181729.1
Pseudomonas reinekei strain MT1 16S ribosomal RNA, partial sequence	Pseudomonas reinekei	2056	2056	93%	0.0	98.87%	1492	NR_042541.1
Pseudomonas glycinae strain MS586 16S ribosomal RNA, complete sequence	Pseudomonas glycinae	2050	2050	93%	0.0	98.79%	1537	NR_179889.1
Pseudomonas vancouverensis strain DhA-51 16S ribosomal RNA, partial sequence	Pseudomonas vancouverensis	2050	2050	93%	0.0	98.79%	1492	NR_041953.1
Pseudomonas izuensis strain IzPS43_3003 16S ribosomal RNA, partial sequence	Pseudomonas izuensis	2045	2045	93%	0.0	98.70%	1459	NR_181014.1
Pseudomonas kribbensis strain 46-2 16S ribosomal RNA, partial sequence	Pseudomonas kribbensis	2043	2043	93%	0.0	98.70%	1492	NR_178854.1
Pseudomonas moraviensis strain 1B4 16S ribosomal RNA, partial sequence	Pseudomonas moraviensis	2039	2039	93%	0.0	98.61%	1524	NR_043314.1

Thus, the Sample RR-3 was identified as *Pseudomonas koreensis*.

Figure 15:BLAST of Sample RR-4 (Forward Sequence)



Job Title	4505948_4M1_27_F
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Program	BLASTN Citation
Database	rRNA_typestrains/16S_ribosomal_RNA See details
Query ID	lcl|Query_57463
Description	4505948_4M1_27_F
Molecule type	dna
Query Length	1111
Other reports	Distance tree of results MSA viewer

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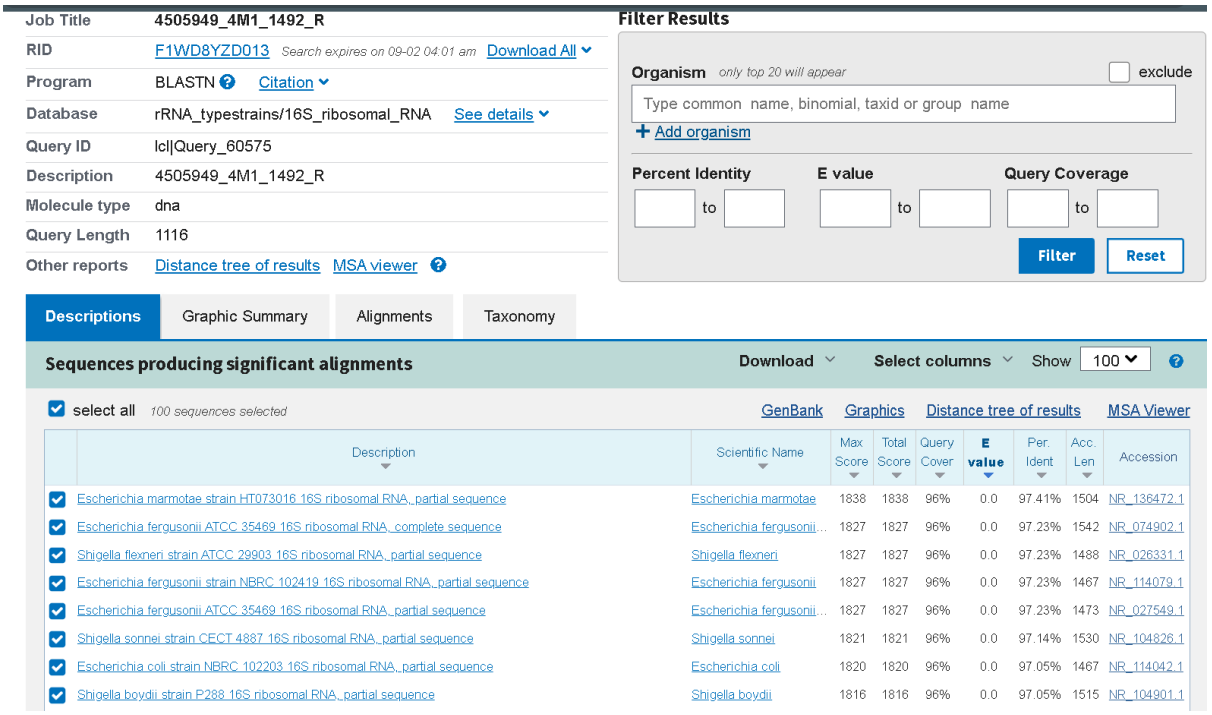
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Shigella flexneri strain ATCC 29903 16S ribosomal RNA, partial sequence	Shigella flexneri	1796	1796	98%	0.0	96.02%	1488	NR_026331.1
Escherichia fergusonii ATCC 35469 16S ribosomal RNA, partial sequence	Escherichia fergusonii ATCC ...	1796	1796	98%	0.0	96.02%	1473	NR_027549.1
Shigella sonnei strain CECT 4887 16S ribosomal RNA, partial sequence	Shigella sonnei	1790	1790	98%	0.0	95.93%	1530	NR_104826.1
Escherichia fergusonii strain NBRC 102419 16S ribosomal RNA, partial sequence	Escherichia fergusonii	1788	1788	98%	0.0	95.84%	1467	NR_114079.1
Escherichia fergusonii ATCC 35469 16S ribosomal RNA, complete sequence	Escherichia fergusonii ATCC ...	1784	1784	98%	0.0	95.84%	1542	NR_074902.1
Escherichia coli strain NBRC 102203 16S ribosomal RNA, partial sequence	Escherichia coli	1783	1783	98%	0.0	95.75%	1467	NR_114042.1
Shigella dysenteriae strain ATCC 13313 16S ribosomal RNA, partial sequence	Shigella dysenteriae	1779	1779	98%	0.0	95.75%	1487	NR_026332.1
Shigella boydii strain P288 16S ribosomal RNA, partial sequence	Shigella boydii	1764	1764	97%	0.0	95.80%	1515	NR_104901.1

Figure 16:BLAST of Sample RR-4 (Reverse Sequence)



Job Title	4505949_4M1_1492_R
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Program	BLASTN Citation
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Query ID	lcl|Query_60575
Description	4505949_4M1_1492_R
Molecule type	dna
Query Length	1116
Other reports	Distance tree of results MSA viewer

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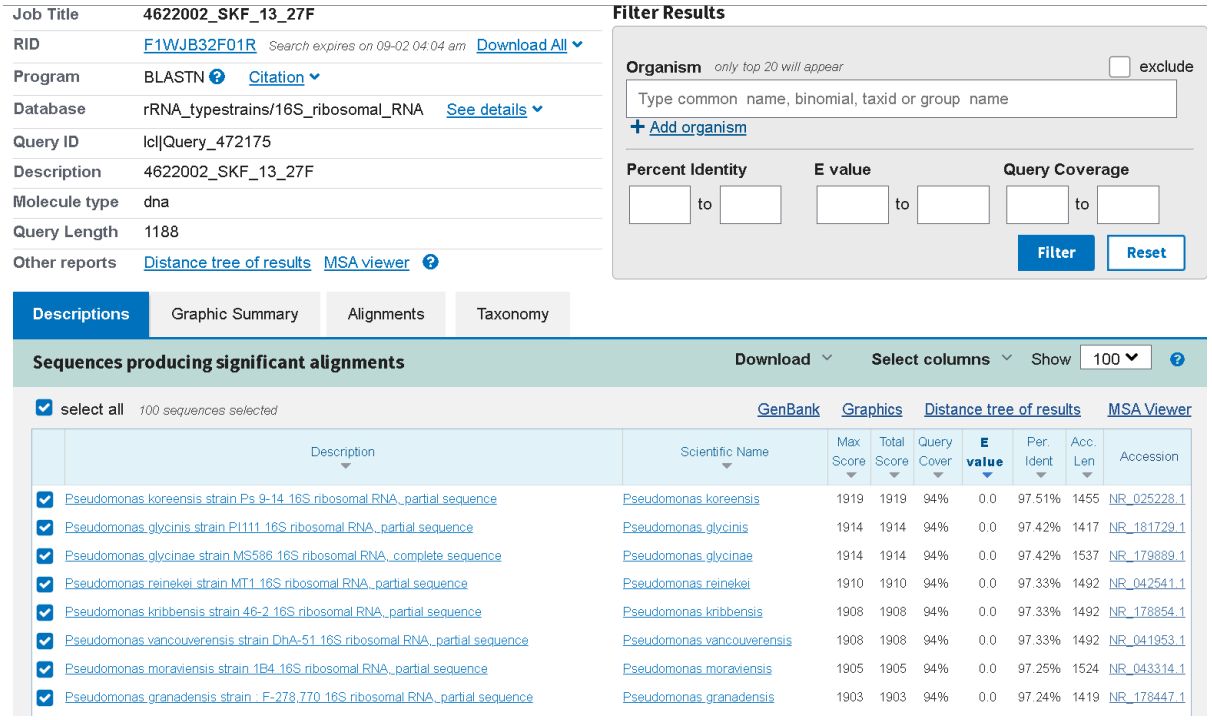
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Escherichia marmotae strain HT073016 16S ribosomal RNA, partial sequence	Escherichia marmotae	1838	1838	96%	0.0	97.41%	1504	NR_136472.1
Escherichia fergusonii ATCC 35469 16S ribosomal RNA, complete sequence	Escherichia fergusonii...	1827	1827	96%	0.0	97.23%	1542	NR_074902.1
Shigella flexneri strain ATCC 29903 16S ribosomal RNA, partial sequence	Shigella flexneri	1827	1827	96%	0.0	97.23%	1488	NR_026331.1
Escherichia fergusonii strain NBRC 102419 16S ribosomal RNA, partial sequence	Escherichia fergusonii	1827	1827	96%	0.0	97.23%	1467	NR_114079.1
Escherichia fergusonii ATCC 35469 16S ribosomal RNA, partial sequence	Escherichia fergusonii...	1827	1827	96%	0.0	97.23%	1473	NR_027549.1
Shigella sonnei strain CECT 4887 16S ribosomal RNA, partial sequence	Shigella sonnei	1821	1821	96%	0.0	97.14%	1530	NR_104826.1
Escherichia coli strain NBRC 102203 16S ribosomal RNA, partial sequence	Escherichia coli	1820	1820	96%	0.0	97.05%	1467	NR_114042.1
Shigella boydii strain P288 16S ribosomal RNA, partial sequence	Shigella boydii	1816	1816	96%	0.0	97.05%	1515	NR_104901.1

Thus, the Sample RR-4 was identified as *Escherichia fergusonii*.

Figure 17:BLAST of Sample RR-5 (Forward Sequence)



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Description	4622002_SKF_13_27F
Molecule type	dna
Query Length	1188
Other reports	Distance tree of results MSA viewer

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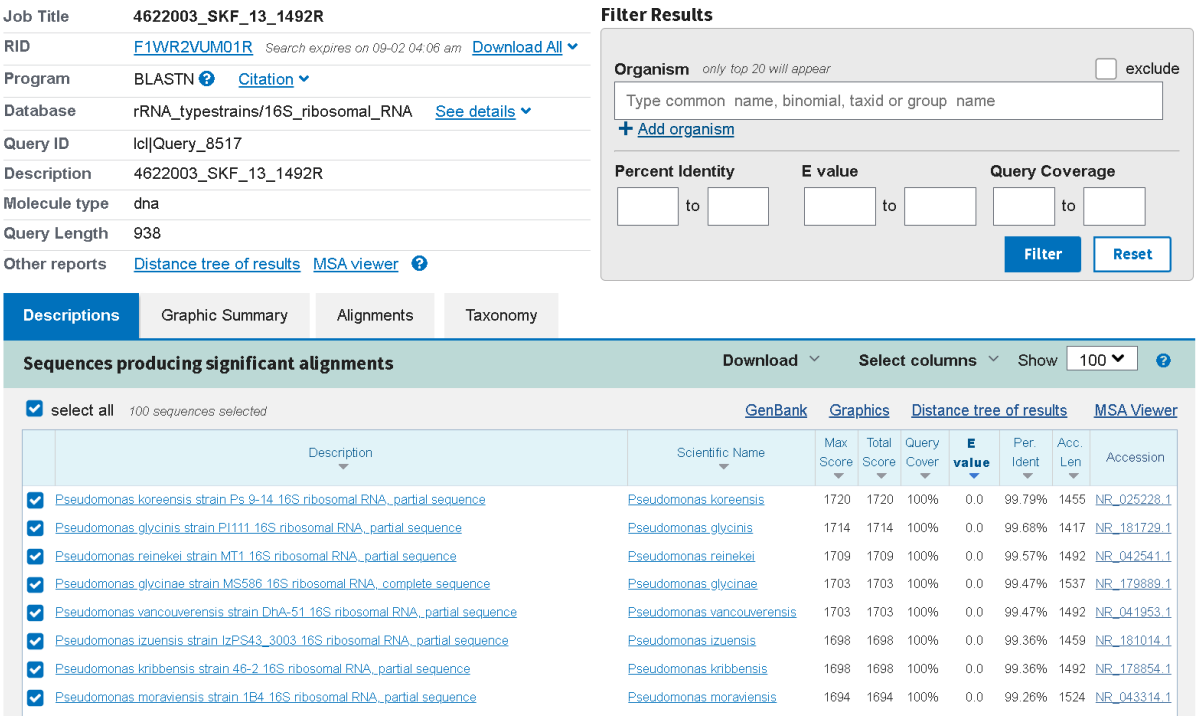
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Pseudomonas koreensis strain Ps 9-14 16S ribosomal RNA, partial sequence	Pseudomonas koreensis	1919	1919	94%	0.0	97.51%	1455	NR_025228.1
Pseudomonas glycinis strain PI111 16S ribosomal RNA, partial sequence	Pseudomonas glycinis	1914	1914	94%	0.0	97.42%	1417	NR_181729.1
Pseudomonas glycinae strain MS586 16S ribosomal RNA, complete sequence	Pseudomonas glycinae	1914	1914	94%	0.0	97.42%	1537	NR_179889.1
Pseudomonas reinekei strain MT1 16S ribosomal RNA, partial sequence	Pseudomonas reinekei	1910	1910	94%	0.0	97.33%	1492	NR_042541.1
Pseudomonas kribbensis strain 46-2 16S ribosomal RNA, partial sequence	Pseudomonas kribbensis	1908	1908	94%	0.0	97.33%	1492	NR_178854.1
Pseudomonas vancouverensis strain DhA-51 16S ribosomal RNA, partial sequence	Pseudomonas vancouverensis	1908	1908	94%	0.0	97.33%	1492	NR_041953.1
Pseudomonas moraviensis strain 1B4 16S ribosomal RNA, partial sequence	Pseudomonas moraviensis	1905	1905	94%	0.0	97.25%	1524	NR_043314.1
Pseudomonas granadensis strain : F-278,770 16S ribosomal RNA, partial sequence	Pseudomonas granadensis	1903	1903	94%	0.0	97.24%	1419	NR_178447.1

Figure 18:BLAST of Sample RR-5 (Reverse Sequence)



Job Title	4622003_SKF_13_1492R
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Program	BLASTN Citation
Database	rRNA_typestrains/16S_ribosomal_RNA See details
Query ID	lcl|Query_8517
Description	4622003_SKF_13_1492R
Molecule type	dna
Query Length	938
Other reports	Distance tree of results MSA viewer

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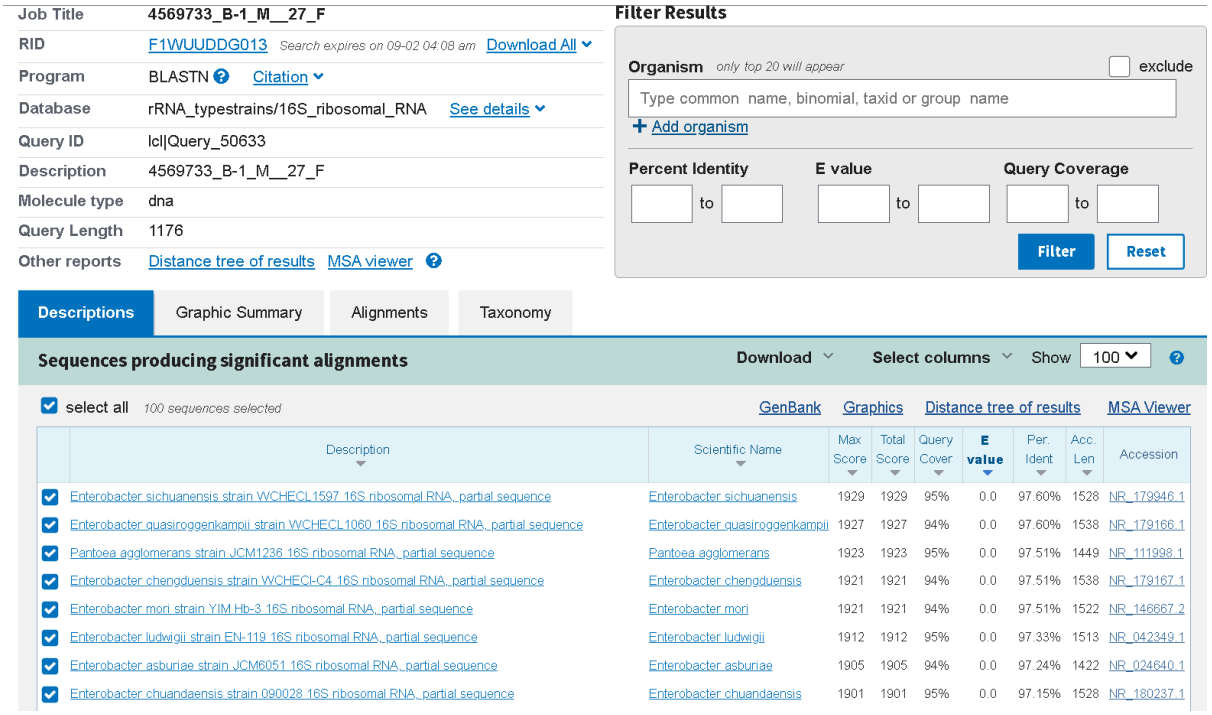
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Pseudomonas koreensis strain Ps 9-14 16S ribosomal RNA, partial sequence	Pseudomonas koreensis	1720	1720	100%	0.0	99.79%	1455	NR_025228.1
Pseudomonas glycinis strain PI111 16S ribosomal RNA, partial sequence	Pseudomonas glycinis	1714	1714	100%	0.0	99.68%	1417	NR_181729.1
Pseudomonas reinekei strain MT1 16S ribosomal RNA, partial sequence	Pseudomonas reinekei	1709	1709	100%	0.0	99.57%	1492	NR_042541.1
Pseudomonas glycinae strain MS586 16S ribosomal RNA, complete sequence	Pseudomonas glycinae	1703	1703	100%	0.0	99.47%	1537	NR_179889.1
Pseudomonas vancouverensis strain DhA-51 16S ribosomal RNA, partial sequence	Pseudomonas vancouverensis	1703	1703	100%	0.0	99.47%	1492	NR_041953.1
Pseudomonas izuensis strain IzPS43_3003 16S ribosomal RNA, partial sequence	Pseudomonas izuensis	1698	1698	100%	0.0	99.36%	1459	NR_181014.1
Pseudomonas kribbensis strain 46-2 16S ribosomal RNA, partial sequence	Pseudomonas kribbensis	1698	1698	100%	0.0	99.36%	1492	NR_178854.1
Pseudomonas moraviensis strain 1B4 16S ribosomal RNA, partial sequence	Pseudomonas moraviensis	1694	1694	100%	0.0	99.26%	1524	NR_043314.1

Thus, the Sample RR-5 was identified as *Pseudomonas koreensis*.

Figure 19:BLAST of Sample RR-6 (Forward Sequence)



Job Title	4569733_B-1_M__27_F
RID	F1WUUDDG013 Search expires on 09-02 04:08 am Download All
Program	BLASTN Citation
Database	rRNA_typestrains/16S_ribosomal_RNA See details
Query ID	lcl|Query_50633
Description	4569733_B-1_M__27_F
Molecule type	dna
Query Length	1176
Other reports	Distance tree of results MSA viewer

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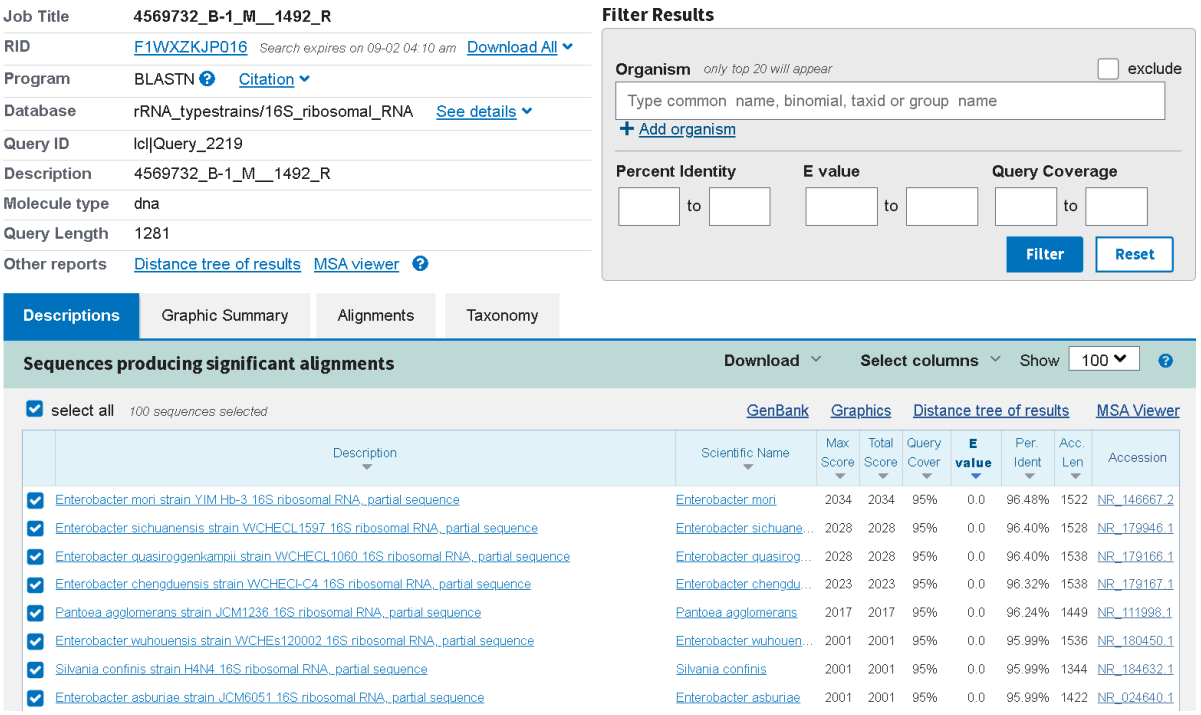
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Enterobacter sichuanensis strain WCHECL1597 16S ribosomal RNA, partial sequence	Enterobacter sichuanensis	1929	1929	95%	0.0	97.60%	1528	NR_179946.1
Enterobacter quasiroggenkampii strain WCHECL1060 16S ribosomal RNA, partial sequence	Enterobacter quasiroggenkampii	1927	1927	94%	0.0	97.60%	1538	NR_179166.1
Pantoea agglomerans strain JCM1236 16S ribosomal RNA, partial sequence	Pantoea agglomerans	1923	1923	95%	0.0	97.51%	1449	NR_111998.1
Enterobacter chengduensis strain WCHECI-C4 16S ribosomal RNA, partial sequence	Enterobacter chengduensis	1921	1921	94%	0.0	97.51%	1538	NR_179167.1
Enterobacter mori strain YIM Hb-3 16S ribosomal RNA, partial sequence	Enterobacter mori	1921	1921	94%	0.0	97.51%	1522	NR_146667.2
Enterobacter ludwigii strain EN-119 16S ribosomal RNA, partial sequence	Enterobacter ludwigii	1912	1912	95%	0.0	97.33%	1513	NR_042349.1
Enterobacter asburiae strain JCM6051 16S ribosomal RNA, partial sequence	Enterobacter asburiae	1905	1905	94%	0.0	97.24%	1422	NR_024640.1
Enterobacter chuandaensis strain 090028 16S ribosomal RNA, partial sequence	Enterobacter chuandaensis	1901	1901	95%	0.0	97.15%	1528	NR_180237.1

Figure 20:BLAST of Sample RR-6 (Reverse Sequence)



Job Title	4569732_B-1_M__1492_R
RID	F1WXZKJP016 Search expires on 09-02 04:10 am Download All
Program	BLASTN Citation
Database	rRNA_typestrains/16S_ribosomal_RNA See details
Query ID	lcl|Query_2219
Description	4569732_B-1_M__1492_R
Molecule type	dna
Query Length	1281
Other reports	Distance tree of results MSA viewer

Filter Results

Organism only top 20 will appear exclude

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Descriptions | Graphic Summary | Alignments | Taxonomy

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Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Enterobacter mori strain YIM Hb-3 16S ribosomal RNA, partial sequence	Enterobacter mori	2034	2034	95%	0.0	96.48%	1522	NR_146667.2
Enterobacter sichuanensis strain WCHECL1597 16S ribosomal RNA, partial sequence	Enterobacter sichuane...	2028	2028	95%	0.0	96.40%	1528	NR_179946.1
Enterobacter quasiroggenkampii strain WCHECL1060 16S ribosomal RNA, partial sequence	Enterobacter quasirog...	2028	2028	95%	0.0	96.40%	1538	NR_179166.1
Enterobacter chengduensis strain WCHECI-C4 16S ribosomal RNA, partial sequence	Enterobacter chengdu...	2023	2023	95%	0.0	96.32%	1538	NR_179167.1
Pantoea agglomerans strain JCM1236 16S ribosomal RNA, partial sequence	Pantoea agglomerans	2017	2017	95%	0.0	96.24%	1449	NR_111998.1
Enterobacter wuhouensis strain WCHEs120002 16S ribosomal RNA, partial sequence	Enterobacter wuhouen...	2001	2001	95%	0.0	95.99%	1536	NR_180450.1
Silvania confinis strain H4N4 16S ribosomal RNA, partial sequence	Silvania confinis	2001	2001	95%	0.0	95.99%	1344	NR_184632.1
Enterobacter asburiae strain JCM6051 16S ribosomal RNA, partial sequence	Enterobacter asburiae	2001	2001	95%	0.0	95.99%	1422	NR_024640.1

Thus, the Sample RR-6 was identified as *Enterobacter Sichuanensis*.

Chapter 4

Discussion

Gram-negative bacterial infections are especially concerning since they are growing increasingly resistant to practically every antibiotic now in use, mirroring pre-antibiotic conditions. The advent of MDR Gram-negative bacteria has had an impact on medical practice in general. It is assumed that any infectious disease can be cured with antibiotics. Antibiotics are produced on a global scale of around 100,000 tons per year, and their usage has had a significant influence on the existence of microorganisms on Earth. More pathogen strains have developed antibiotic resistance, and some have developed resistance to several antibiotics and chemotherapeutic drugs, a condition known as multidrug resistance. (Nikaido, 2009) When compared to monotherapy, potential benefits of antibiotic combinations include a larger antibacterial range, synergistic effects, and a lower likelihood of resistance forming during therapy. The use of a combination antibiotic regimen for Gram-negative sepsis is debatable. Combinations are increasingly being used to augment the antibacterial properties of existing medications against multidrug-resistant pathogens in the lack of evidence-based therapeutic choices. Excessive usage of combinations, on the other hand, should be avoided since it may raise the risk of toxicity, superinfections, selection of resistant strains, and greater costs. (Tängdén, 2014) Antibiotic resistance has progressively developed over time, creating a critical need for safer alternatives to antibiotics, especially ones that are natural, non-toxic, and do not create resistance. One such possibility is Vitamin C (ascorbic acid), a natural antioxidant component that has no adverse effects, is affordable, and is freely available. Vitamin C is a water-soluble vitamin that the body cannot generate, thus it must be supplied regularly. Green

peppers, red peppers, strawberries, tomatoes, broccoli, brussels sprouts, turnip, Indian gooseberry, and other leafy vegetables are high in Vitamin C (Hassuna et al., 2023)

Ciprofloxacin is a commonly accessible and cheap antibiotic that is used to treat a range of bacterial illnesses in Bangladesh. It is a quinolone antibiotic. It acts by inhibiting bacterial growth. This antibiotic exclusively cures bacterial diseases such as pneumonia, gonorrhea, typhoid fever; a severe infection frequents in underdeveloped countries, infectious diarrhea, and skin, bone, joint, abdominal, and prostate infections. Ciprofloxacin is also useful in the treatment of bronchitis, sinus infections, and urinary tract infections. (*Ciprofloxacin: MedlinePlus Drug Information*, n.d.)

In the present study, three antibiotics were chosen against which all our bacterial samples were resistant according to the Antibiotic Susceptibility Tests. These were Gentamicin, Ceftazidime, and Ciprofloxacin. Ceftazidime falls under the class of Beta Lactams and Ciprofloxacin is a Fluoroquinolone. We carried out individual MICs of these antibiotics in BHI media inoculated with our bacterial samples and individual MICs of Vitamin C in BHI media inoculated with the same strains of bacteria. Using the results of their MICs, we carried out MIC experiments using different combination dosages of Ceftazidime + Vitamin C and Ciprofloxacin + Vitamin C. In the case of the combination experiments of Ceftazidime and Vitamin C, clear broths were found which showed that the combination of Ceftazidime and Vitamin C could inhibit the growth of bacteria. Furthermore, the results of Ciprofloxacin + Vitamin C MIC value showed that their combination could inhibit the growth of the bacterial samples. Thus, the antibiotics Ceftazidime and Ciprofloxacin were chosen for further phases of MIC experiments in combination with Vitamin C. The experiments were carried out using the $C_1V_1 = C_2V_2$ formula. Following the MIC, the FICI of these combination dosages was calculated for both Ceftazidime + Vitamin C and Ciprofloxacin + Vitamin C combinations. According to the FICI formula, the

FICI of the Ceftazidime+Vitamin C combination for all the strains was greater than 0.5 which indicated that the combination of Ceftazidime and Vitamin C were not synergistic. However, the FICI of the Ciprofloxacin +Vitamin C combination for all the strains was within 0.5 which proved that their combination was synergistic. By simply combining a previously resistant drug with Vitamin C, it was feasible to reduce the minimum antibiotic concentration necessary to inhibit bacterial growth. This drew attention to the need to combine antibiotics with Vitamin C.

According to our results, the combination of Ciprofloxacin and Vitamin C works synergistically against *Pseudomonas koreensis, Escherichia fergusonii, and Enterobacter sichuanensis* since all of their respective FICIs of Ciprofloxacin and Vitamin C were within 0.5. In support of the results, several studies were found. For instance, according to a study by A.A.H. Al Qushawi and K. J. Al-Ruaby in 2021, The disc diffusion technique was used to assess the antibiotic susceptibility of 54 *Pseudomonas aeruginosa* bacterial isolates, and the findings indicated that Ciprofloxacin was the most effective antibiotic against the bacterial isolates. Twelve isolates were chosen to assess the impact of ascorbic acid when coupled with antibiotics utilizing the disk diffusion technique. Ascorbic acid was employed at various dosages ranging from 1 to 22.2 mg. The findings revealed that there is a synergistic interaction between Vitamin C and the majority of antibiotics. Furthermore, the synergistic impact rises with increasing Vitamin Concentration. Furthermore, another study by Hassuna et al., 2023 showed how Vitamin C works as an antibacterial and anti-biofilm agent against uropathogenic E. coli (UPEC) strains. The antibacterial and anti-biofilm properties of Vitamin C against uropathogenic E. coli strains were evaluated in vivo utilizing a urinary tract infection (UTI) rat model. The effective concentration of Vitamin C required to prevent the development of the majority of the study isolates (70%) was 1.25 mg/mL. Vitamin C had a synergistic impact with the majority of the antibiotics tested; no antagonistic effect was seen. Moreover, Amábile-

Cuevas (2023) stated in their article that Ascorbate and Antibiotics, at Concentrations Attainable in Urine, Can Inhibit the Growth of Resistant Strains of Escherichia coli Cultured in Synthetic Human Urine. The impact of 10 mM ascorbate (which is not inhibitory on its own) combined with antibiotics was evaluated against resistant isolates of Escherichia coli from lower urinary infections in Mueller-Hinton broth and synthetic human urine. These findings imply that 10 mM ascorbate can boost antibiotic inhibitory action against resistant bacteria in urine. According to Aburawi et al. (2013), an investigation was carried out to see if Vitamin C inactivates Gram-positive (S. aureus ATCC 29213) and Gram-negative (*E. coli* ATCC 25922) bacterial populations alone or in combination with Penicillin G. In *E. coli* populations, Vitamin C showed a similar antibacterial impact to Penicillin G (120mg), and this effect was considerably ($p>0.05$) boosted with the three Penicillin G dosages used. As a result, Vitamin C enhanced the impact of the Penicillin G antibiotic to the point where a combined sub-effective dose of Penicillin G (30mg) with Vitamin C (10mg) had the same effect as 120 mg Penicillin G. This study found that Vitamin C plays an important role in bacterial population inactivation and can increase the action of particular antibiotics by using them at lower concentrations, which may help decrease some unwanted side effects. Lastly, Kwiecińska-Piróg et al. (2019) stated that they investigated the effect of ascorbic acid combined with antibiotics on *P. mirabilis'* ability to build a biofilm. The susceptibility of the strains was assessed using EUCAST guidelines. Spectrophotometric analysis was used to assess the effect of ascorbic acid (0.4 mg mL1) in conjunction with antibiotics on biofilm development. The addition of ascorbic acid to the culture medium reduced the inhibitory effect of fluoroquinolones, allowing *P. mirabilis* strains to form biofilms. However, the addition of ascorbic acid during aminoglycoside therapy may interfere with the treatment of urinary tract infections caused by *P. mirabilis* biofilm.

Therefore, this study proves that the combination of Ciprofloxacin and Vitamin C is effective against multi-drug-resistant *Pseudomonas koreensis, Escheichia fergusonii, and Enterobacter sichuanensis*. Ciprofloxacin and Vitamin C work synergistically, according to this in vitro study. Combining antibiotics with Vitamin C is not currently practiced in hospitals and the rules for doing so are also ambiguous. Several studies have been conducted on the use of various combination regimens for treating these Gram-negative bacteria, all of which are particularly antibiotic resistant, although these studies generally lacked in vivo validation. It is currently unknown which antimicrobial agent/class combinations work best for treating resistant infections.

Chapter 5

Conclusion

In conclusion, it is imprudent to overlook the seriousness of antibiotic-resistant microorganisms, like *Pseudomonas koreensis*, *Enterobacter sichuanensis* and *Escherichia fergusonii*. These pathogens are rapidly acquiring mechanisms to resist treatment. However, if we can enhance the effectiveness of existing antibiotics by synergizing them with complementary agents like Vitamin C, it could potentially be a life-saving and economically viable solution.

Furthermore, as the incidence of antibiotic-resistant infections continues to surge in developing nations, including our own, the combination of antibiotics with Vitamin C emerges as a potential lifeline. The synergy between antibiotics and Vitamin C holds the potential to revolutionize the battle against antibiotic-resistant infections. When combined, these two agents can amplify their individual effects, creating a more formidable defense against resilient bacteria such as *Pseudomonas koreensis*, *Enterobacter sichuanensis a*nd *Escherichia fergusonii*. This innovative combination strategy capitalizes on Vitamin C's multifaceted benefits to enhance the effectiveness of antibiotics, offering a novel approach to addressing the growing challenge of antibiotic-resistant infections.

The study's outcomes show the potential of combining antibiotics with Vitamin C to decrease the dosage of antibiotics needed for tackling resistant infections. This significantly influences the feasibility of combining therapies to tackle resilient bacteria such as *Pseudomonas koreensis*, *Enterobacter sichuanensis* and *Escherichia fergusonii*. The future of combination treatment against multi-drug-resistant Gram-negative bacteria may be significantly impacted by these studies.

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