

MULTIDRUG- RESISTANT PROFILE OF ENVIRONMENTAL ISOLATES OF PSEUDOMONAS AERUGINOSA

Submitted in partial fulfilment of the requirements for the
award of Bachelor of Science in Biotechnology

By

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SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)
CATEGORY -1 UNIVERSITY BY UGC

**Accredited with Grade “A++” by NAAC | 12B Status by UGC | Approved by
AICTE**

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DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the Bonafide work of **PEARLINE GRACE G (41300028), SUNITHA ARAVA. A (41300048)** who have done the Project work entitled "**Multidrug- Resistant Profile Of Environmental Isolates Of Pseudomonas Aeruginosa**" under our supervision from October 2023 to April 2024.

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ABSTRACT

Understanding the multi-drug resistance profiles of environmental *Pseudomonas aeruginosa* isolates is crucial for assessing the risk of transmission to clinical settings and for informing strategies to mitigate the spread of antibiotic resistance. This study aimed to characterize the multi-drug resistance profiles of environmental isolates of *Pseudomonas aeruginosa* obtained from various sources. Sample preparation involved serial dilution and collection using standard microbiological techniques. Biochemical tests, including gram staining, oxidase test, citrate test, catalase test, indole test, methyl red test, and motility test, were performed for species differentiation. Isolates were cultured on nutrient agar and *Pseudomonas* cetrimide agar. Antibiotic susceptibility tests were conducted using clinical *pseudomonas* drugs, including ciprofloxacin (5mg), cefotaxime (30mg), azithromycin (15mg), ceftazidime (30mg), and chloramphenicol (5mg). The results revealed a concerning prevalence of multi-drug resistance among the environmental isolates, with notable variations in resistance profiles across different geographical locations and sources. Moreover, molecular techniques were employed to elucidate the genetic mechanisms underlying resistance, shedding light on the spread and evolution of resistance determinants in environmental settings. The study focused on isolates from diverse environmental sources, including metro station cooperative water and hospital water for human consumption, as well as hospital water. Overall, the research underscores the urgency of addressing multi-drug resistance in environmental *Pseudomonas aeruginosa* isolates to prevent their transmission to clinical settings and the consequent challenges in managing infections.

Keywords: Geographic Variations, Molecular Mechanisms, Antibiotic Resistance, Environmental Transmission, Healthcare-Associated Infections.

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LIST OF ABBREVIATIONS

MDR	Multidrug Resistance
SDS	Sodium dodecyl sulphate
ICU	Intensive care unit
NICU	Neonatal intensive care unit
OPD	Outpatient department
ES β L	Extended- spectrum β - lactamse
MBL	Metallo β -lactamase
CTX-M	Hydrolytic activity against cefotaxime

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CHAPTER-1

INTRODUCTION

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa, versatile and antibiotic-resistant, poses a challenge in nosocomial infections. This study was conducted to determine the incidence of *Pseudomonas aeruginosa* in certain environmental samples well as to determine their susceptibility patterns to some commonly used antibiotics and to increase their awareness. These organisms are isolated from corporation water in metro stations and hospitals which are mainly used for human consumption. *Pseudomonas aeruginosa* or P aeruginosa is a gram-negative bacterium that is abundant in the environment intends too opportunistically infect high-risk individuals and is well known for its multi-drug resistance making it hard to treat. (Ikechukwu Benjamin moses *et al.*,2015)

Pseudomonas aeruginosa is an encapsulated gram-negative rod bacterium that's an obligate aerobe so it uses oxygen from metabolism through cellular respiration. It doesn't ferment lactose and it doesn't make spores, but it is catalase, citrate and oxidase positive. It has a flagellum kind of like a tail at one end for motility and it has multiple hair-like appendages called pili all over to help with adhesion to other cells, it also has several multi-drug efflux pumps that efficiently pump medications out of the bacteria making it resistant to a variety of antibiotic. *Pseudomonas aeruginosa* is a resilient pathogen known for its resistance to multiple antibiotics, posing significant challenges in healthcare settings. While clinical isolates have been extensively studied, less attention has been given to environmental sources, which could also harbour multi-drug resistant strains. This study aims to explore the resistance profiles of environmental *Pseudomonas aeruginosa* isolates, shedding light on their prevalence, diversity, and genetic mechanisms of resistance. Understanding these dynamics is crucial for devising effective strategies to combat antibiotic resistance. This research is essential for comprehensively understanding the scope and impact of antibiotic resistance in environmental settings, thus informing strategies to mitigate its spread and safeguard public health. (Ikechukwu Benjamin moses *et al.*,2015)

1.2 Epidemiology and Prevalence of *Pseudomonas aeruginosa*:

Pseudomonas aeruginosa is one of the leading causes of nosocomial infections worldwide, with a growing concern regarding its emergence in environmental reservoirs. Studies have highlighted its prevalence in various ecological niches, including water sources, soil, plants, and biofilms. The ability of *P. aeruginosa* to persist in diverse environments contributes to its transmission and acquisition of resistance determinants, leading to the emergence of MDR strains. (Marin Kollef.,2021)

1.3 Mechanisms of Antibiotic Resistance in *Pseudomonas aeruginosa*

P. aeruginosa exhibits intrinsic resistance to many antibiotics due to its impermeable outer membrane, efflux pumps, and production of β -lactamases and other resistance mechanisms. Additionally, the acquisition of mobile genetic elements, such as plasmids and integrons, facilitates the dissemination of resistance genes within *P. aeruginosa* populations. The interplay between intrinsic and acquired resistance mechanisms contributes to the MDR phenotype observed in environmental isolates. (Maria Fernandez-Billon *et al.*,2022)

1.4 Environmental Factors Driving Multi-Drug Resistance

Environmental factors, including exposure to antibiotics, heavy metals, disinfectants, and biocides, play a significant role in selecting for MDR strains of *P. aeruginosa*. Antibiotic pollution in water bodies and soil can exert selective pressure on environmental bacteria, promoting the survival and proliferation of MDR strains. Furthermore, the presence of biofilms in natural and man-made environments provides a reservoir for the exchange of genetic material, facilitating the spread of antibiotic resistance determinants. (Samreen *et al.*,2019)

1.5 Clinical Implications of Environmental Isolates:

Environmental isolates of *P. aeruginosa* harbor resistance genes that can be horizontally transferred to clinical strains, posing a serious threat to public health. The genetic relatedness between environmental and clinical isolates underscores the importance of surveillance and monitoring programs to track the spread of MDR strains and implement appropriate infection control measures. Strategies aimed at reducing environmental contamination and prudent use of antibiotics are essential for mitigating the emergence and dissemination of MDR *P. aeruginosa*. (MariaVictoria Grosso-Becerra *et al.*,2014)

1.6 Future Directions and challenges

Further research is needed to elucidate the complex interactions between *P. aeruginosa* and its environment, as well as the genetic mechanisms driving antibiotic resistance. Integrated approaches combining genomics, metagenomics, and environmental surveillance can provide valuable insights into the dynamics of MDR *P. aeruginosa* populations. Additionally, the development of alternative therapeutic strategies, such as phage therapy and antimicrobial peptides, may offer promising solutions for combating infections caused by MDR strains. (Shugang Qin *et al.*,2022)

CHAPTER-2

LITERATURE SURVEY

Pseudomonas aeruginosa is a ubiquitous environmental bacterium known for its remarkable adaptability and resistance to multiple antimicrobial agents. With its ability to survive in diverse environments, including soil, water, and hospital settings, *P. aeruginosa* poses a significant threat as an opportunistic pathogen, particularly in immunocompromised individuals and those with cystic fibrosis. Understanding the multi-drug resistance (MDR) profile of environmental isolates of *P. aeruginosa* is crucial for devising effective strategies to combat infections caused by this organism. This literature review aims to provide insights into the current understanding of the MDR profile of environmental isolates of *P. aeruginosa*. (Ikechukwu Benjamin Moses and Ugbo Nnabuike.,2015).

The study focused on antibiotic resistance in *Pseudomonas aeruginosa* and *Escherichia coli* from clinical samples, noting their high resistance to amoxicillin but lower resistance to gentamicin, ofloxacin, and ciprofloxacin. Plasmid curing using sodium dodecyl sulphate (SDS) reduced resistance in some bacteria but led to resistance in others at higher concentrations. Comparison between urban and rural hospitals revealed similar resistance patterns, indicating a widespread issue of antibiotic resistance across different healthcare settings. The research highlights the pressing need for antimicrobial resistance surveillance to inform appropriate antibiotic prescribing practices and combat the growing threat of multidrug-resistant bacterial infections. The findings underscore the complexity of antibiotic resistance mechanisms, with plasmid dynamics playing a crucial role. Efforts to mitigate resistance must consider the potential unintended consequences of treatments like plasmid curing. (Ikechukwu Benjamin Moses and Ugbo Nnabuike.,2015).

The study aimed to document antibiotic resistance in *Pseudomonas aeruginosa* strains isolated from clinical samples over 18 months in a hospital setting (November 2009–April 2011). Samples were collected from nosocomial (wards, cabins, ICU,

NICU) and community (OPD) sources. Out of 368 *P. aeruginosa* strains, 201 (54.62%) were nosocomial, and 167 (45.38%) were from the community, with 25.14% of community strains exhibiting extended-spectrum β -lactamase (ES β L) activity. ES β L strains were also found in nosocomial settings, comprising 26.86% of all nosocomial isolates. The study observed a progressive increase in drug resistance among *P. aeruginosa* strains to 15 antibiotics tested. (Mahesh *et al.*,2012)

P. aeruginosa infections are a significant concern due to their high mortality rates, especially among vulnerable patient groups. The study highlighted the prevalence of multidrug-resistant (MDR) strains, posing challenges for effective clinical management. Factors contributing to the spread of MDR pathogens included inappropriate antibiotic use, irregular dosing, and lack of awareness among healthcare workers and the public. The study emphasized the need for robust surveillance systems, awareness campaigns, and judicious antibiotic prescribing practices to combat the escalating issue of antibiotic resistance in healthcare settings. (Mahesh *et al.*,2012)

The study aimed to investigate the prevalence of multidrug-resistant *Pseudomonas aeruginosa* in different regions of Iran. Through systematic database searches and inclusion criteria, 23 relevant studies were selected for meta-analysis. The pooled incidence of MDR *P. aeruginosa* was estimated to be 58%, indicating a substantial prevalence of antibiotic resistance in Iranian hospitals. The highest prevalence of MDR *P. aeruginosa* was found in Tehran (100%), while Zahedan showed the lowest prevalence at 16%. Resistance rates against various antibiotics were also assessed, with ceftazidime and amikacin showing the highest resistance rates at 50%, followed by piperacillin/tazobactam at 49%. In contrast, imipenem exhibited the lowest resistance rate at 31%. The study revealed significant heterogeneity in the prevalence of MDR *P. aeruginosa* across different regions of Iran, highlighting the complex nature of antibiotic resistance patterns. Publication bias was detected in the analysis, indicating a potential limitation in the available literature. (Hamid Vaez *et al.*,2018)

The study investigated the antibiotic resistance patterns of *Pseudomonas aeruginosa* isolates from various aquatic sites in Greece. Among 245 confirmed *P. aeruginosa* isolates, resistance profiles were analysed, with a focus on extended spectrum β -lactamases (ESBLs) and integron genes associated with antibiotic resistance. Initially, 52% of isolates were classified as wild, 16.3% as non-wild (susceptible to most antibiotics except specific ones), and 32% as resistant (showing resistance or intermediate resistance to more than three antibiotics). Different resistance profiles were identified, including those related to intrinsic resistance mechanisms of *P. aeruginosa* such as AmpC derepression (R1), increased efflux (R2), and loss of OprD (R3). Notably, ESBL producers were isolated from various sources, including mains water and swimming pools, indicating the presence of antibiotic-resistant strains in diverse aquatic habitats. The study also screened isolates for the presence of class 1 and class 2 integron genes, which are genetic elements capable of capturing and expressing antibiotic resistance genes. Thirteen isolates were positive for the class 1 integron gene, with variable regions containing genes conferring resistance to aminoglycosides. None of the isolates tested positive for the class 2 integron gene. Further molecular analysis revealed the presence of the CTX-M group 9 β -lactamase gene in three isolates, indicating the emergence of ESBL genes in Greek aquatic environments. While phenotypically ESBL and metallo β -lactamase (MBL) positive isolates did not show positive results in molecular tests, the detection of CTX-M group 9 β -lactamase highlights the potential transmission of ESBL genes in aquatic ecosystems. (Pappa Olga *et al.*, 2016)

CHAPTER-3

AIM AND OBJECTIVES

3.1 Aim

This research contribute to the study of antibiotic resistance patterns and guide strategies for addressing to public health concerns associated with multidrugresistant bacteria.

3.2 Objectives

- Assessing the Spectrum and Degree of Resistance.
- Identification Prevalent Resistance Patterns.
- Characterizing Resistance Mechanism.
- Informing Antibiotic Stewardship.
- Contributing to Public Health Strategies by increasing awareness.

CHAPTER-4

MATERIALS AND METHODS

4.1 Sample collection

We have obtained samples from two distinct sources: metro water and hospital water.

4.2 Materials

Agar plates for culturing *Pseudomonas aeruginosa* isolates, various antibiotics for testing drug resistance, sterile swabs for sample collection, incubators for bacterial growth, spectrophotometers for measuring bacterial density, and PCR equipment for genetic analysis.

4.3 Sample processing

Process the samples to isolate *Pseudomonas aeruginosa* strains using selective and differential media, such as cetrimide agar, to encourage the growth of the target bacteria while inhibiting others.

4.3.1 Isolation and Identification

Environmental samples collected from both metro water and hospital water undergo processing to isolate *Pseudomonas aeruginosa* strains utilizing selective and differential media, notably cetrimide agar. Following isolation, the strains are identified through biochemical tests including the oxidase test, catalase test, indole test, methyl red test, and Voges-Proskauer test. (Ugbo Emmanuel *et al.*, 2015)

4.3.2 Stock preparation

Prepare stock solutions by diluting the isolated strains in autoclaved distilled water. 1 ml of sample added to 99ml of autoclaved distilled water. 2 stocks were prepared for the sample of 100ml.

4.3.3 Serial Dilution

Perform serial dilutions of the stock solutions to create a range of concentrations. This is typically achieved by diluting a portion of the stock solution with a known volume of distilled water multiple times. 9 ml of the sample was mixed with 1 ml of distilled water, and this process was repeated three times for both samples.

4.4 Biochemical tests

All requisite biochemical tests, including the oxidase, catalase, indole, methyl red, and Voges-Proskauer tests, have been diligently conducted. These tests serve as fundamental procedures in confirming the identity of *Pseudomonas aeruginosa* isolates. The oxidase test aids in identifying bacteria with cytochrome c oxidase activity, a characteristic feature of *P. aeruginosa*. Similarly, the catalase test assists in distinguishing bacteria that produce catalase enzymes, another trait commonly found in *P. aeruginosa*. Furthermore, the indole test helps ascertain the ability of the isolates to metabolize tryptophan, providing additional evidence for their identification. The methyl red test evaluates the ability of the bacteria to perform mixed acid fermentation, further corroborating the presence of *P. aeruginosa*. Lastly, the Voges-Proskauer test aids in detecting acetoin production, aiding in the differentiation of *P. aeruginosa* from other organisms. Through meticulous execution of these biochemical tests, a comprehensive understanding of the identity and characteristics of the *Pseudomonas aeruginosa* isolates has been achieved, laying a solid foundation for subsequent analyses. (Ugbo Emmanuel *et al.*, 2015)

4.5 Antimicrobial Susceptibility Testing

Standardized methods, such as disk diffusion, broth microdilution, or agar dilution, are used to determine the susceptibility of *P. aeruginosa* isolates to a panel of antimicrobial agents, including antibiotics from different classes.

- ciprofloxacin (5mg)
- cefotaxime (30 mg)
- azithromycin (15mg)
- ceftazidime (30mg)
- chloramphenicol (5mg)

4.6 Data Analysis and Interpretation

Statistical analyses are performed to correlate antimicrobial resistance phenotypes with genotypic resistance determinants and environmental factors.

Data interpretation involves identifying trends, associations, and potential risk factors contributing to the emergence and spread of MDR *P. aeruginosa* in environmental reservoirs.

By employing these methods in combination, researchers can obtain a comprehensive understanding of the MDR profile, mechanisms, and genetic determinants of environmental isolates of *P. aeruginosa*, contributing to the development of effective strategies for surveillance, infection control, and antimicrobial stewardship. (Etinosa O. Igbinosa *et al* ., 2012)

4.7 Minimum Bactericidal Concentration (MBC)

4.7.1 Preparation of Sample

Tetracycline drug was mixed with dimethyl sulfoxide (DMSO) to create a 2ml sample. (Etinosa O. Igbinosa *et al* ., 2012)

4.7.2 Control Blank

A blank sample was kept as a control, presumably containing only DMSO or a similar solvent without the antibiotic.

4.7.3 Test Tubes with Nutrient Broth

A set of 6 test tubes containing nutrient broth was prepared.

4.7.4 Bacterial Inoculation

Bacterial concentration was pipetted into each test tube. It seems like you used decreasing volumes of bacterial suspension for each sample to create a gradient of bacterial concentrations:

- Sample 1st: 1000ul
- Sample 2nd: 500ul
- Sample 3rd: 250ul
- Sample 4th: 125ul
- Sample 5th: 70ul
- Sample 6th: 20ul

4.7.5 Incubation

After inoculating the bacterial suspension, the test tubes were incubated for 24 hours.

4.7.6 Smear Preparation

After incubation, the concentrations from each test tube were used to create smears on petri dishes containing nutrient broth. The petri dishes were labelled accordingly, with one labelled as the control (presumably with the original bacterial concentration without any antibiotic).

4.8 Molecular Analysis (with minor changes)

Employ molecular techniques such as polymerase chain reaction (PCR) or sequencing to investigate the genetic mechanisms underlying resistance.

4.8.1 Identification of microorganism by sanger sequencing method

4.8.1.1 Sample Information

Sample details: Culture samples in Plate

Test parameter: Microbial Identification by sequencing method

Test method: Sanger sequencing method

4.8.1.2 Methods Involved

1. DNA Isolation Protocol
2. Gel Electrophoresis and Quality Check
3. PCR Amplification
4. Column Purification
5. Sanger Sequencing
6. Data Analysis

4.8.1.3 DNA Isolation protocol

- Initial Sample Preparation-Take a 1ml sample in an Eppendorf tube.
- First Centrifugation-Centrifuge the sample at 4000rpm for 5 minutes. Decant the supernatant, leaving behind the pellet.
- Lysis and Extraction-To the pellet tube, add 100 µl of PBS, 400µl of Cell Lysis Buffer, and 5µl of proteinase. Incubate the tube at 55°C for 1-2 hours.
- Phenol Extraction-Add 500µl of saturated phenol to the sample and mix well for 5 minutes. Centrifuge at 12000 rpm for 10 minutes. Transfer the supernatant to a new tube.
- Phenol-Chloroform-Isoamyl Alcohol Extraction-Add 25:24:1 of Phenol: chloroform: Isoamyl alcohol to the tube. Mix for 5 minutes by inverting. Centrifuge at 12000 rpm for 10 min. Transfer the supernatant to a new tube.
- Chloroform Extraction-Add 500µl of chloroform to the tube. Mix for 5 minutes by inverting. Centrifuge at 12000 rpm for 10 minutes. Transfer the aqueous layer carefully to a new tube.
- Precipitation and Washing-Add an equal volume of 100% Ethanol or Isopropanol to the aqueous layer. Keep at -20°C for 1-2 hours. Centrifuge at 14000 rpm for 20 minutes. Decant the supernatant.
- Washing and Drying-Add 1 ml of 70% Ethanol to the pellet. Centrifuge at 14000 rpm for 10 minutes. Decant the supernatant. Air dry the pellet at room temperature to remove droplets.
- Elution-Add 50 - 60µl of Elution buffer to the dried pellet.

4.8.1.4 Gel Electrophoresis and Quality check

The quantity was measured using Nano Drop Spectrophotometer and the quality was determined using 1.5 % agarose gel. A single band of high-molecular weight DNA has been observed.

4.8.1.5 PCR Amplification

The microbial DNA was amplified using universal primers for microbial identification by PCR from the above isolated DNA.

A single discrete PCR amplicon band was observed when resolved on Agarose Gel.

The PCR product was purified to remove contaminants.

Primer for Bacterial identification

27 F	AGAGTTTGATCCTGGCTCAG
1492 R	CGGTTACCTTGTTACGACTT

4.8.1.6 Purification by Column

PCR products were purified by inhouse column purification kit method. (QIAquick spin., 2020)

PROCEDURE :

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample, and then mix. It is not necessary to remove mineral oil or kerosene. For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil). Spin Handbook 01/2020 25

2. If pH Indicator I has been added to Buffer PB, check that the mixture's color is yellow. If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The mixture will turn yellow.

3. Prepare the vacuum manifold and columns according to step 3a or 3b.

4. 24 Plus (see Figure 7, page 39): Insert up to 24 spin columns into the luer extensions of the 24 Plus. Close unused positions with luer caps, and then connect 24 Plus to a vacuum source.

5. Other vacuum manifolds: Follow the supplier's instructions. Insert each column into a luer connector.

6. To bind DNA, load the samples into the columns by decanting or pipetting, and then apply vacuum. After the samples have passed through the column, switch off the vacuum source. The maximum loading volume of the column is 800 µl. For sample volumes greater than 800 µl, simply load again.

7. To wash, add 0.75 ml of Buffer PE to each column and apply vacuum.

8. Transfer each column to a microcentrifuge tube or the provided 2 ml collection tubes. Centrifuge for 1 min at 17,900 x g (13,000 rpm).

IMPORTANT: This spin is necessary to remove residual ethanol (Buffer PE).

9. Place each column into a clean 1.5 ml microcentrifuge tube

4.8.1.7 Sequencing

The purified PCR amplicon was sequenced using either of the Universal forward and reverse primers

Sequencing was done using BDT v3.1 Cycle sequencing kit on ABI 3500 Genetic Analyzer.

CHAPTER- 5

Results and discussion

5.1 Hospital and metro corporate water sample



Fig 5.1 Streaking of metro corporate and hospital sample

After incubation, the bacterial growth from the previous step was inoculated and streaked onto another set of cetrimide agar plates. This step aims to isolate and identify *Pseudomonas aeruginosa* bacteria based on their ability to grow on cetrimide agar, which is selective for *Pseudomonas* species. The streaking technique involves spreading the bacterial sample evenly across the agar surface in a pattern that allows for the isolation of individual colonies. These colonies can then be further analysed using biochemical tests or molecular methods to confirm their identity as *Pseudomonas aeruginosa*. This process helps in studying the antibiotic resistance patterns and prevalence of *Pseudomonas aeruginosa* in the sample under investigation. (Jvo Siegrist., 2007)

5.2 Inoculation

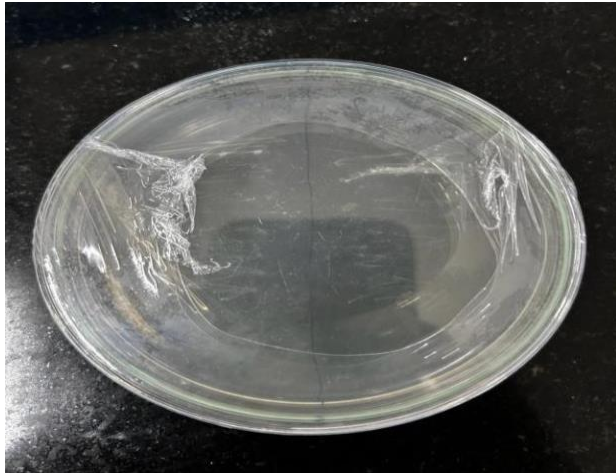


Fig 5.2.1 Metro sample

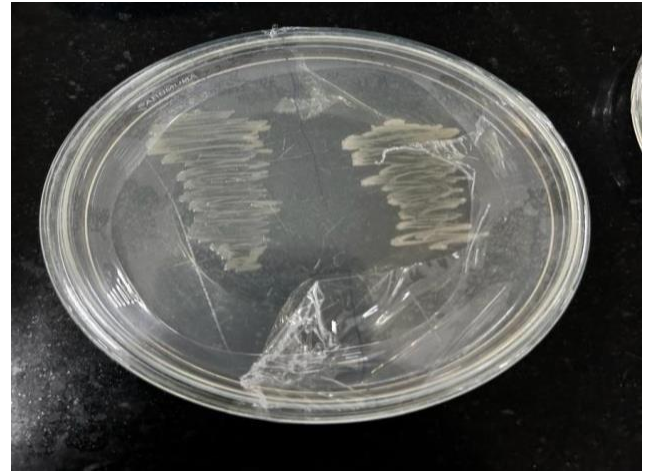


Fig 5.2.2 Hospital sample

In inoculating water samples from metro corporate and hospital environments for *Pseudomonas aeruginosa*, the focus lies on understanding the prevalence and potential risks associated with this pathogen. Robust sampling strategies covering various locations and isolation techniques using selective media and molecular methods are employed to accurately identify and confirm *Pseudomonas aeruginosa* isolates. Assessing antibiotic resistance and virulence factors helps gauge their threat level. Ultimately, these efforts inform public health measures, such as improving water quality management and implementing infection control in healthcare settings, to mitigate the risks posed by this versatile and potentially harmful bacterium. (Jvo Siegrist., 2007)

5.3 Biochemical test:

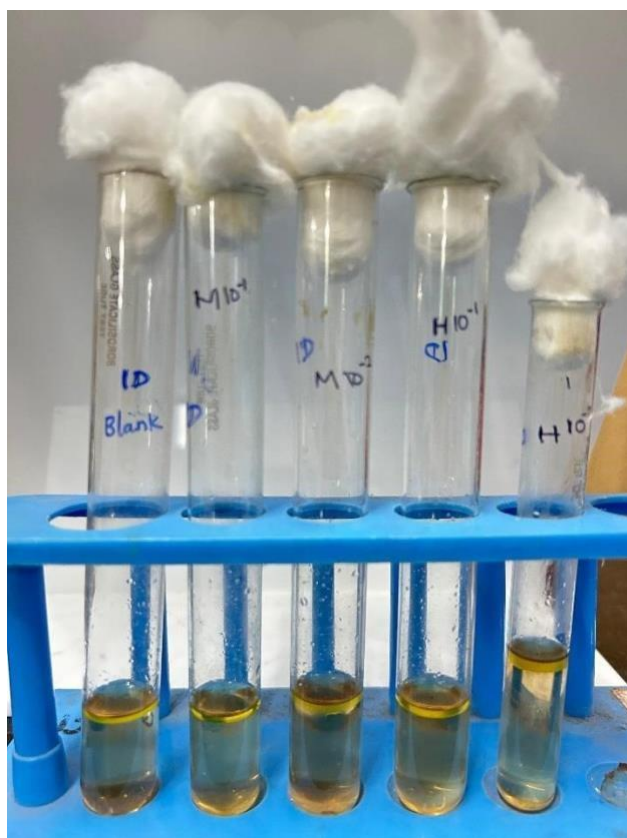


Fig 5.3.1 Indole test

The negative results in the indole test for the hospital sample and metro sample was partially positive which had a yellowish color indicate that the bacteria in these samples do not possess the enzyme tryptophanase necessary for converting tryptophan into indole, pyruvic acid, and ammonia. This finding was the same from the article but since it was done in a clinical setting the results of metro was assumed to be opposite or with partial results, the results suggesting a potential absence or low activity of tryptophanase. It's essential to consider the implications of this result in terms of bacterial identification and classification, as well as its potential impact on biochemical pathways within these bacterial populations. (Ugbo Emmanuel et al.,2015)

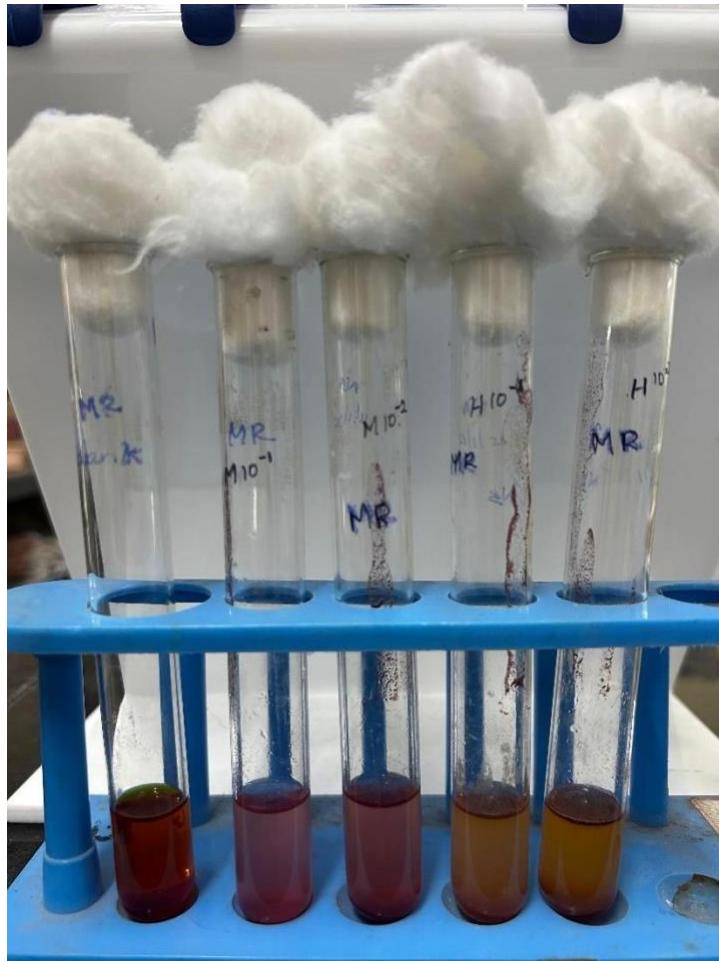


Fig 5.3.2 Methyl red test

The negative results of the methyl red test for the hospital sample and metro samples gave a partially light pink color suggest that it can have other spp. and the hospital strain lack significant glucose fermentation capabilities, leading to minimal acid production. This finding was the same from the article but since it was done in a clinical setting the results of metro was assumed to be opposite or with partial results and the metabolic differences within *P. aeruginosa* strains and underscores the importance of further exploring the genetic and environmental factors that influence their metabolic profiles. Understanding these metabolic nuances can have implications for clinical treatment strategies and infection control measures, particularly in hospital settings where *P. aeruginosa* infections are a concern. (Ugbo Emmanuel et al., 2015)

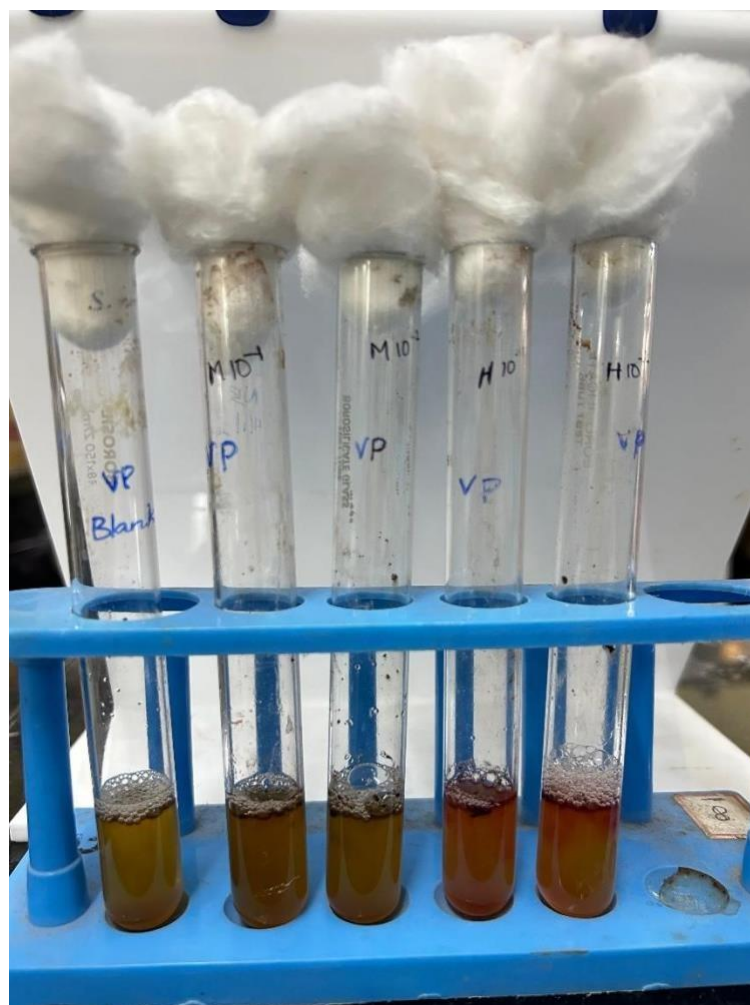


Fig 5.3.3 Voges-Proskauer test

A negative VP test result in *Pseudomonas aeruginosa* in hospital sample which was reddish brown in color compared to metro sample which was yellowish brown in color signifies its inability to ferment glucose to produce acetoin, distinguishing it from other bacterial species that can undergo this metabolic process. This characteristic regarding the metro sample will be the opposite or partially different stemmed from the fact that the study was conducted in a clinical setting despite the findings aligning with those of the article. the metabolic trait plays a crucial role in differentiating *Pseudomonas aeruginosa* from other bacteria during biochemical identification schemes, contributing to accurate species classification. (Ugbo Emmanuel et al.,2015)

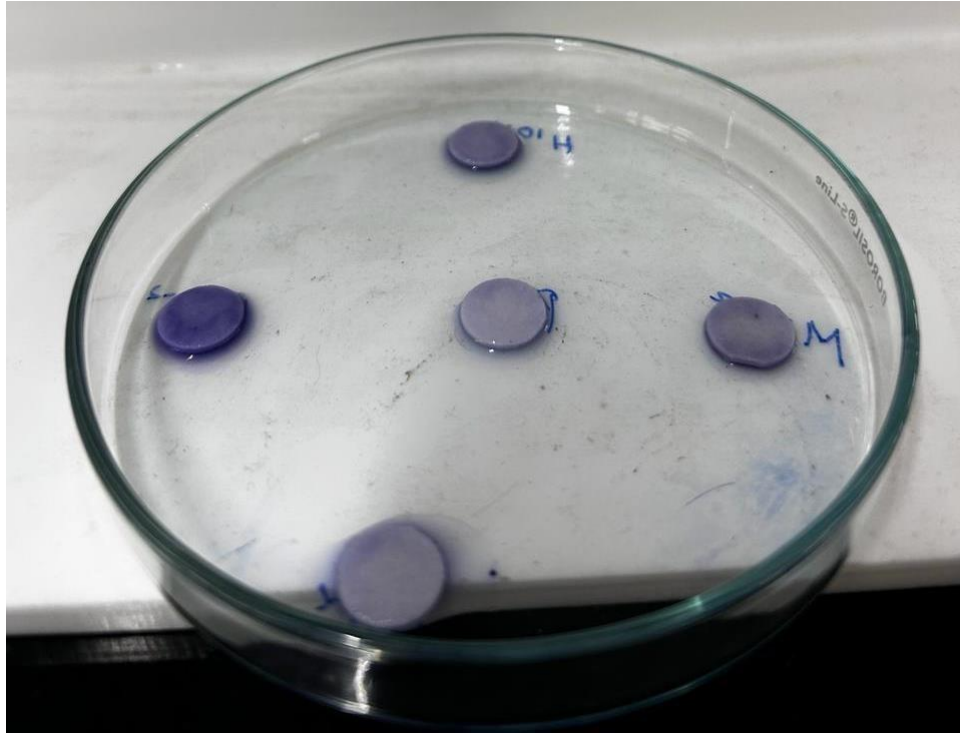


Fig 5.3.4 Oxidase test

The positive oxidase test results in both samples confirm the presence of cytochrome c oxidase activity, aligning with the expected biochemical profile of *Pseudomonas aeruginosa* where the hospital sample had higher color opacity than the metro sample. This information that the outcome of the metro study might contradict or vary to some extent due to the research being carried out in clinical environment, despite the results matching those discussed in the article for hospital sample which contributes to the accurate identification and differentiation of bacterial species, aiding in clinical and research settings where precise microbial characterization is essential. (Ugbo Emmanuel et al.,2015)

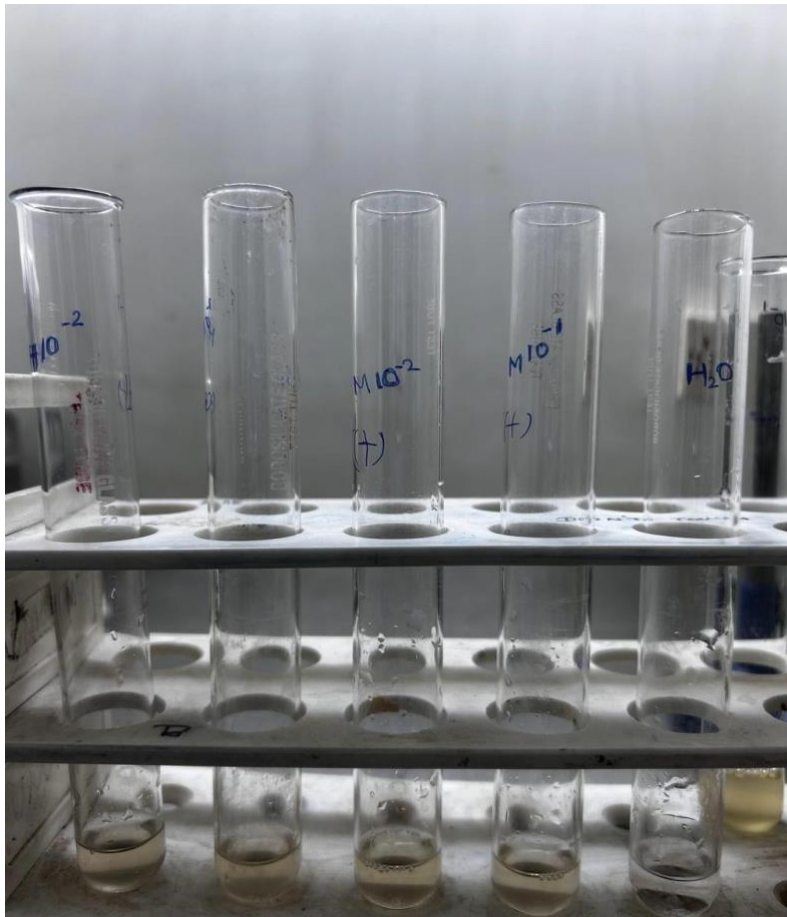


Fig 5.3.4 Catalase test

The positive catalase test results in the metro sample which gave bubbles in form of reaction and hospital samples didn't show any reaction indicate the presence of catalase enzyme activity. This information that the metro sample outcome was assumed to be opposed based on the difference in results on the clinical setting, despite the results aligning for the hospital sample with those mentioned in the article which plays a crucial role in converting hydrogen peroxide into water and oxygen, a process that protects bacteria from oxidative damage. In the case of *Pseudomonas aeruginosa*, being catalase-positive allows it to effectively neutralize hydrogen peroxide and other reactive oxygen species, contributing to its survival and virulence. These results are significant for identifying and understanding the biochemical capabilities of bacterial species, particularly in contexts where oxidative stress is a factor. (Ugbo Emmanuel et al.,2015)

5.4 Minimum Bactericidal Concentration (MBC) test



Fig 5.4.1 Drug inoculation (Tetracycline)

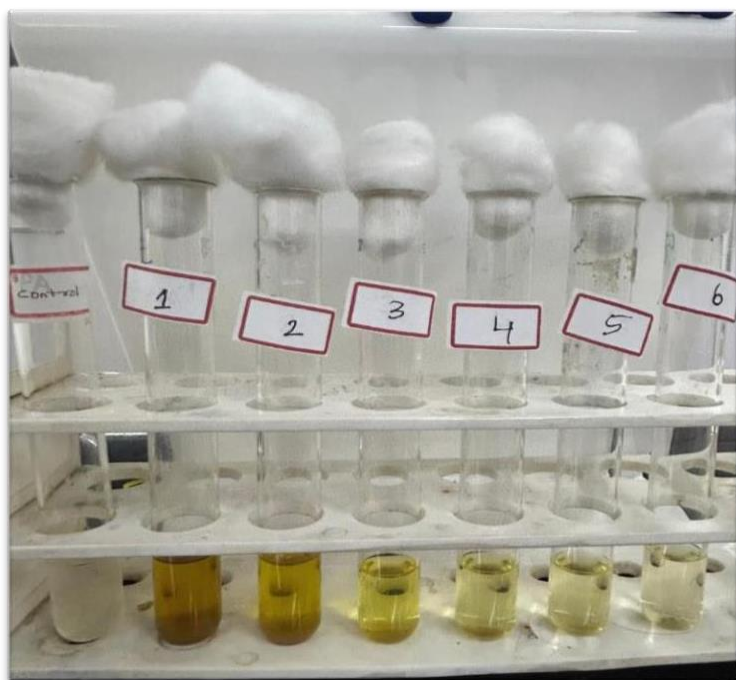


Fig 5.4.2 After 24 hours of inoculation

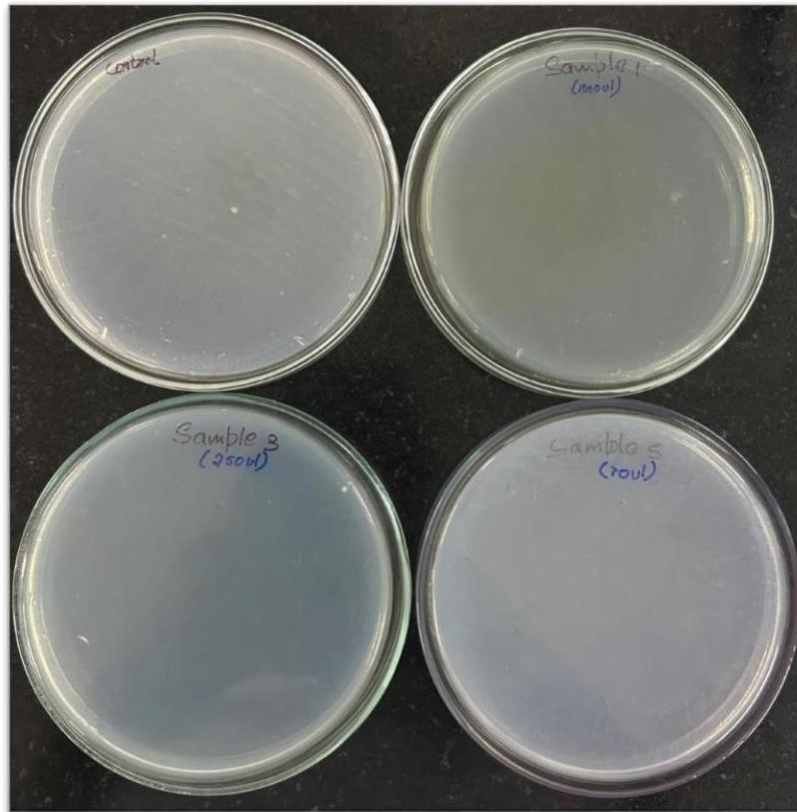


Fig 5.4.3 Spread plate after inoculation

The Minimum Bactericidal Concentration (MBC) test protocol involved preparing a sample of tetracycline mixed with dimethyl sulfoxide (DMSO) and setting up a control blank with just DMSO. Six test tubes with nutrient broth were then prepared, and decreasing volumes of bacterial suspension were added to create a gradient of bacterial concentrations. After incubation for 24 hours, smears were prepared from each concentration on petri dishes containing nutrient broth, with one dish serving as the control without antibiotic. This methodology allows for the determination of the minimum concentration of tetracycline needed to kill bacteria, providing valuable information on the drug's effectiveness against the tested bacterial strain. (Mahjabeen Khan *et al.*, 2020)

5.5 SPECTROMETER READING

Table 5.5.1 Spectrometer Reading

Concentration	SAMPLE 1 st mg/mL	SAMPLE 2 nd mg/mL	SAMPLE 3 rd mg/mL	SAMPLE 4 th mg/mL	SAMPLE 5 th mg/mL	SAMPLE 6 th mg/mL	CONTROL
Observation at 600nm	0.098	0.107	0.116	0.131	0.149		0.276

5.6 Data analysis & Interpretation for DNA Sequencing :(pseudomonas aeruginosa)

TTATCGCGTTAGCTTGCGATACTGAGAGCTCAAGGGCCCCAACGGCTATCTA
CCTCCTTTAAGGGGTGGAATAACAAGGGATTTTATCCCGGTTTGCTCCCCAC
CCTTTCCTGCCCAATGGCAATATTTGTCCAGGGGGCCGCCTTCCCCAATGGT
GTTCTTCCTAATCTACCCATTTACCGCTACACCGGAAATTCCACCACCCTC
TACCGTACTCTAACTCGCCAGTTTTGGATGCAGTTCCCAGGGTGAACCCGGG
GATTTACATCCGACTTGATAAAACCCCTACGCGCGCTTTACGCCCAATAATT
CCCATTAACGCTTGACCCCTTCCTATTACCGCGGCTGCTGGCAGCAAATTA
CCGGGGCTTATTCTGTTGGGAACGTCAATACCACCGGGTATTAACCTACTGG
CCTTCCTCCCCACTTAAAGTGCTTTACAATCCCAAGAGCTTCTTCACACACGC
CGCATGGCTGGATCAAGCTTTCCCCCATTGTCCAATATTCCCCACTGCTGCCT
CCCGTAAGAATCTGGACCGTGTCTCAGTTCCAGTGTGGCTGATCATTCTCTCA
GAACAACCTACGGGATCGTCGCCCTGGTGAGCCTTTACCCACCCACTAGCTA
ATCCACCTACGCTCATCTGATAGCGTGAGGTCCGAAAGATCCCCCTTTTCT
CCCTCAAGACGTATGCCGGTATTAGCGCCCGTTTCCGGACGTTATCCCCCA
CTACCAAGCAGATTCCCGATGCATTACTCACCCGTCCGCCACTCAATCCCCG
AGCAGCTCCTCTCATGCTGCTCGACTTGATGTGTTAGTCTGCCGCCAGCGT
TCAATCT

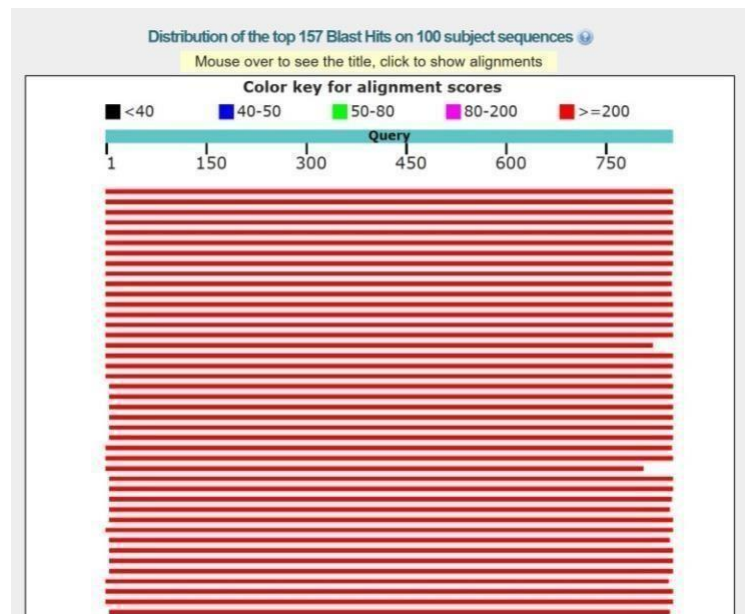


Fig 5.6.1 Color key for alignment score

- Based on maximum identity score and organism similarity query coverage sequences were selected for further analysis.

Sequences producing significant alignments:

Select: [All](#) [None](#) Selected:0

[Alignments](#) [Download](#) [GenBank](#) [Graphics](#) [Distance tree of results](#)

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☐	Pseudomonas aeruginosa strain JCM 5962 16S ribosomal RNA gene, partial sequence	994	994	100%	0.0	88.00%	MK796437.1
☐	Pseudomonas paraeruginosa strain PA7 16S ribosomal RNA gene, partial sequence	994	994	100%	0.0	88.00%	MG757383.1
☐	Pseudomonas aeruginosa strain JCM5962(T) 16S ribosomal RNA gene, partial sequence	994	994	100%	0.0	88.00%	KX946966.1
☐	Pseudomonas aeruginosa DSM 50071, complete genome	994	3978	100%	0.0	88.00%	CP012001.1
☐	Pseudomonas aeruginosa genome assembly NCTC10332, chromosome : 1	994	3978	100%	0.0	88.00%	LN831024.1
☐	Pseudomonas aeruginosa strain DSM 50071 16S ribosomal RNA, partial sequence	994	994	100%	0.0	88.00%	NR_117678.1
☐	Pseudomonas aeruginosa strain JCM 5962(T) 16S ribosomal RNA gene, partial sequence	994	994	100%	0.0	88.00%	MW009677.1
☐	Pseudomonas aeruginosa strain ATCC 10145 16S ribosomal RNA, partial sequence	994	994	100%	0.0	88.00%	NR_114471.1
☐	Pseudomonas aeruginosa partial 16S rRNA gene, type strain DSM 50071T	990	990	99%	0.0	87.97%	LN681564.1
☐	Pseudomonas aeruginosa strain NBRC 12689 16S ribosomal RNA, partial sequence	990	990	99%	0.0	87.97%	NR_113599.1
☐	Pseudomonas aeruginosa gene for 16S ribosomal RNA, partial sequence, strain: JCM 5962	990	990	99%	0.0	87.97%	LC069033.1
☐	Pseudomonas aeruginosa strain DSM 50071 16S ribosomal RNA gene, partial sequence	989	989	100%	0.0	87.90%	KT825518.1
☐	Pseudomonas aeruginosa strain JCM 5962 16S ribosomal RNA gene, partial sequence	972	972	100%	0.0	87.54%	MT740443.1

Fig 5.6.2 Sequences producing significant alignment

- Using NCBI software and EMBL the sequence distance was calculated and the phylogenetic tree was construct by fast minimum evolution and neighbor joining method

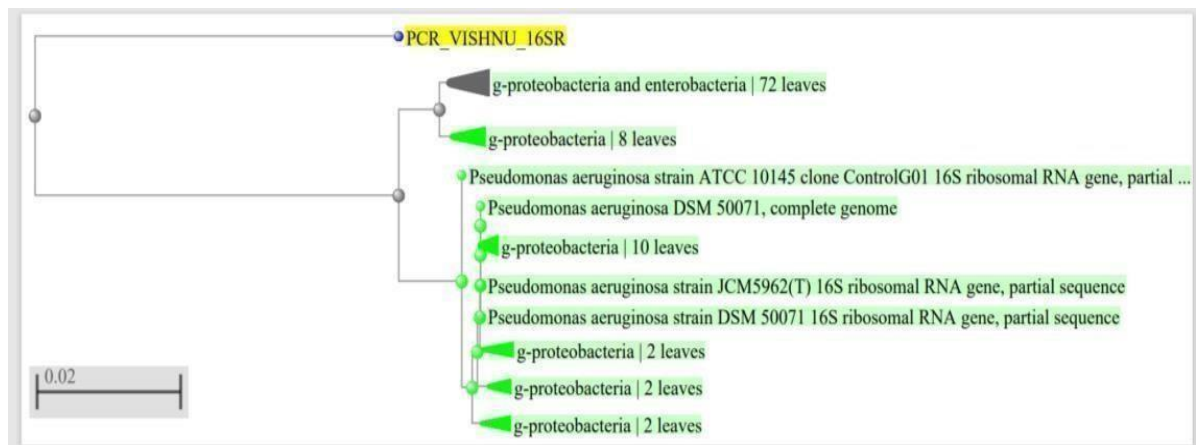


Fig 5.6.3 molecular identification of pseudomonas aeruginosa

Based on sequence homology and phylogenetic analysis, the Microbial culture, was considered to be *Pseudomonas* sp. 16S ribosomal RNA gene.

The molecular analysis procedure described follows a systematic approach to identify microorganisms using the Sanger sequencing method. It begins with DNA isolation from a sample, including centrifugation, lysis, and multiple extractions to obtain purified DNA. Quality checks through gel electrophoresis confirm the presence of high-molecular-weight DNA. PCR amplification with universal primers produces a single discrete amplicon, which is then purified to remove impurities. Finally, sequencing using universal primers on a genetic analyzer allows for accurate microbial identification. This methodical process ensures reliable results in investigating the genetic mechanisms underlying resistance in microorganisms (Mohamed A. Hussain *et al.*, 2023)

CHAPTER-6

SUMMARY AND CONCLUSION

The study aimed to understand the multi-drug resistance profiles of environmental *Pseudomonas aeruginosa* isolates, particularly focusing on samples from metro station corporation water and hospital water. Standard microbiological techniques were employed for sample preparation, including serial dilution, biochemical tests for species differentiation, and antibiotic susceptibility tests using clinical drugs. The results indicated a concerning prevalence of multi-drug resistance among the environmental isolates, with variations in resistance patterns across different geographical locations and sample sources. Molecular techniques were also utilized to elucidate genetic mechanisms underlying resistance, providing insights into the spread and evolution of resistance determinants in environmental settings.

After conducting a comparative study between metro corporate water and hospital water samples, we have found that the metro corporate water sample demonstrates a better quality characteristics than the hospital water sample. This conclusion is based on various parameters such as purity, microbial content, and overall suitability for consumption or industrial use. The biochemical tests indicates metro water had lower concentration of *Pseudomonas aeruginosa* than compared to hospital and overall was more pure than hospital which indicates a cleaner water for consumption for humans and other uses. Hospital samples was highly contaminated with higher concentration of *Pseudomonas aeruginosa* which can be cause infection if consumed frequently.

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