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DRUG-RESISTANT PATTERN AND BIOFILM CHARACTERISTICS OF PSEUDOMONAS AERUGINOSA ISOLATED FROM CLINICAL SAMPLES

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ABSTRACT

Pseudomonas aeruginosa (P. aeruginosa) is an organism of one health concern. With a high fatality rate, it is an important cause of nosocomial infections in immunocompromised individuals. This study looked into the potential causes of urinary tract infections (UTIs) in hospitalized patients, including *P. aeruginosa* biofilm formation and drug resistance patterns. In the ongoing study, 105 urine and pus samples from suspected male and female patients were collected from different hospitals, and they were then inoculated on Blood MacConkey and selective Cetrimide Agar for the isolation of *P. aeruginosa*. Gram's staining was used to identify these isolated strains before biochemical analyses. Disks containing the antibiotics Meropenem (100 mg), Azithromycin (15 mg), Amikacin (30 mg), Gentamicin (30 mg), Aztreonam (30 mg), and Enrofloxacin (5 mg) were used in disc diffusion antimicrobial susceptibility testing. Antibiotic susceptibility was examined using Muller Hinton Agar and findings were obtained by measuring the diameter of the zones surrounding the discs in accordance with CLSI standards. Out of 105 clinical samples, 63 samples were positive for *P. aeruginosa*. Out of 63 positive, 36 and 27 isolates were positive for females and males, respectively. Overall percentage positivity was 60%. Females showed highest percent positivity, 57.14% while in males it was 42.85%. Patients within the age of 40 60 showed highest percentage positivity. All isolates showed 100% resistance against

Azithromycin while Amikacin was resistant in few isolates. Gentamicin, Aztreonam, Meropenem, and Enrofloxacin were found as effective drugs. Positive isolates were further processed for biofilm formation using 96 96-well plate or a micro-titration plate. Out of 63 positive samples, 45 were biofilm-producing, having OD value more than 0.2. Most of them were strong biofilm producers. Our results concluded that *P. aeruginosa* was a common cause of UTIs and catheter-associated infections in hospitalized patients, and most of these *P. aeruginosa* strains are biofilm-producing and multidrug-resistant.

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative opportunistic bacterium that can cause significant infections in vulnerable individuals, such as individuals who have cystic fibrosis and persistent conditions as well as burn patients and patients who are hospitalized in an intensive care unit (ICU) (Killough et al., 2022). Additionally, *P. aeruginosa* can result in a wide range of urinary tract infections, burn wounds, blood circulation, and respiratory tract problems (Sanya et al., 2023). The most prevalent bacterial infections in hospitals and the general population in humans are urinary tract infections (Belete et al., 2019). UTIs are inflammatory disorders of the urinary system commonly brought on by aberrant and excessive pathogenic bacterial development (Prasada Rao et al., 2022). Hospital-acquired infections like catheter-associated infections have emerged as the most prevalent and significant cause of morbidity in clinics and hospitals, accounting for nearly 35% of nosocomial infections and the second most frequent cause of bacteremia in hospitalized patients (Okonko et al. 2019; Pachori et al., 2019). Depending on the circumstances of the infection urinary tract infections can either be acute or persistent (Lee et al., 2019). Patients with weakened immune systems are more susceptible to infection and bacteria can easily colonize the bladder lining and cause urinary tract infections (Saleem and Daniel, 2021). Overuse of anti-pseudomonal medicines have raised the danger of diseases brought on by *P. aeruginosa* (Barp et al., 2023). The expression of efflux pumps, the inhibition of enzyme synthesis, and the creation of biofilms, among other antimicrobial mechanisms, have made it difficult to treat bacterial infections (Nasser et al., 2020). Antibiotic resistance in *P. aeruginosa* is caused by a number of causes including the production of various enzymes that modify antibiotics such as extended spectrum lactamases metallo lactamases and aminoglycoside modifying enzymes (Kanj and Kanafani, 2011) (Chatterjee et al. 2016). Nearly 30% of all nosocomial *P. aeruginosa* isolates across different nations were metallo beta lactamases positive (Manoharan et al., 2010). Hospital acquired infections brought on by *P. aeruginosa* that are resistant to numerous medications have become a severe problem in clinical care settings as the advent of multi-drug resistance MDR strains has grown (Pachori et al. 2019). *P. aeruginosa* is too hard to eradicate in healthcare associated infections using simply antibiotic therapy (Spagnolo et al., 2021). As *P. aeruginosa* like many other bacteria, often form biofilms which are adaptive biofilm styles of growth which are typically triggered by contact with a surface or stress are incredibly resistant to physical rupture and cell death brought on by environmental obstacles (Chadha et al., 2022). Hospital settings are becoming more concerned due to biofilm toughness resistance to currently available antibiotics and host immunological clearance processes (Taylor et al., 2014). Because of their innate resistance to antimicrobial treatments, biofilm is accountable for chronic infections that persist (El Fouly et al. 2015). *P. aeruginosa* uses

several genes to produce biofilms such as pel, psl, and alg (Chung et al., 2023). Production of the *P. aeruginosa* biofilm is a well-controlled process that goes through several stages (Sarkar, 2020). Its development is influenced by a variety of factors with two component systems (TCSs) being a significant one (Mikkelsen et al., 2011). The development various pathogenic components, like type 3 enzyme secretory mechanisms, and a reduction in the susceptibility for bacterial membranes throughout the development of biofilms give microorganisms resistance to certain antibiotic classes (Qin et al., 2022). The most noticeable extracellular pigment produced by *P. aeruginosa* is phenazine. The main distinguishing feature of *P. aeruginosa* is the development of a water soluble blue green phenazine molecule and soluble Pyocyanin pigment (Sakhtah et al., 2016). Therefore, *P. aeruginosa* can cause persistent infections and survive in hard environments like hospitals because to biofilm production (Borchaloee and Hashemi, 2023). In order to successfully manage severe infections brought on by biofilm formation in *P. aeruginosa* strains, it is important to understand the association between bacterial genotype and biofilm phenotype (Redfern et al., 2021). Regarding the major contribution of *P. aeruginosa* of infections acquired in hospitals and the absence of thorough investigation on these findings, we aimed to check the occurrence of *P. aeruginosa* from clinical samples of humans and antibiotic resistance pattern of *P. aeruginosa* isolated from clinical human samples and to determine biofilm forming ability of *P. aeruginosa* isolated from human clinical samples.

Materials and methods

Ethical consent

The present study was approved by Institutional BioSafety/ BioEthics Committee and University of Agriculture Faisalabad (IBC, UAF). Informed consent was obtained from all individuals or their caregivers prior to sampling and use of samples for additional laboratory analysis.

Isolation and identification of *P. aeruginosa*

A total of 105 human clinical samples (Urine Pus) of patients (both Male and Female) were collected in urine containers, catheter bags, and sterile swabs. Cetrimide agar was used for the isolation and cultivation of *P. aeruginosa*. This medium inhibits the growth of other bacterial species as it contains ammonium salt and acts as a selective medium for the *P. aeruginosa* because it produces certain pigments to help it grow in the presence of detergents such as ammonium salt. Standard microbiological and biochemical tests such as colony characteristics, oxidase test, citrate utilization, etc., were carried out for identification of *P. aeruginosa* isolates.

Cultivation and Purification of *P. aeruginosa*

The prepared Blood and MacConkey media were used for the growth of the causative organism. Inoculation of human clinical samples was done by swabbing the samples on plates and incubated the plates in an inverted position for 24 hours at 37°C. After incubation, results and colony characteristics were determined by the appearance of growth on the media. For the purification of *P. aeruginosa,* Cetrimide agar media was prepared and poured into plates. Suspected growth was picked and streaked on the plates. Four streaks were done on the plates and placed plates in incubator for 24 hours at 37°C. The purified growth was preserved in glycerol at 20°C.

Microscopic Examination and Biochemical Characterization of *P. aeruginosa*

In order to identify *P. aeruginosa,* Gram's staining was used. Gram-positive cells have a thick layer of peptidoglycan on their cell walls, whereas Gram-negative cells have a thin layer. This difference permits the

crystal violet iodine combination to be maintained after staining in Gram-positive cells. After staining, the slides were examined under a microscope at 100x objective lens for the confirmation of *P. aeruginosa*. By using Bergey's Manual of Systematic Bacteriology biochemical test was performed for the phenotypic identification of *P. aeruginosa.*

Antibiotic susceptibility testing

The inhibition zone pattern was used to assess *P. aeruginosa* susceptibility to several types of antimicrobial drugs in Muller Hinton's agar using the conventional disc diffusion method. The Kirby Bauer technique was used to assess *P. aeruginosa* antibiotic sensitivity and resistance patterns. To identify isolates as susceptible, the zone diameter interpretive criteria for *P. aeruginosa* were employed. The antipseudomonal antibiotics tested include Meropenem (l0µg), Azithromycin (15 µg), Amikacin (30 µg), Gentamicin (30 µg), Aztreonam (30 µg), and Enrofloxacin (5 µg) from Oxoid Ltd, Basingstoke Hampshire England. Results were determined by measuring diameter of zones around the discs according to Clinical and Laboratory Standards Institute (CLSI) guidelines.

Detection of Biofilm formation

According to established techniques, microtiter plate-based biofilm quantification experiments were carried out utilizing adherent cell staining with crystal violet. *P. aeruginosa* strains from overnight cultures were subcultured at 37°C in 96-well microtiter plates at an optical density (OD) OD600 of 0.2. Planktonic cells were removed after 24 hours of development and the wells were then washed with PBS. Biofilms were then fixed in 100 µl of 99 percent (v/v) cold methanol before being stained with 0.1 percent (v/v) crystal violet. After washing, 150µl of glacial acetic acid (33 percent v/v) was used to solubilize the crystal violet. Thermo Multiskan FC reader was used to measure the absorbance at 550 nm. Three times the assay was done in hexaplicate.

Statistical analysis

Percent positivity of *P. aeruginosa* isolated from urine sample was determined by following formula:

$$\text{Percentage Positivity} = \frac{\text{no. of positive samples}}{\text{no. of total samples}} \times 100$$

Results

The primary purpose of this study was isolation, purification and determination of antibiograms and biofilm formation of *P. aeruginosa* isolated from different patients of different wards. The characteristics and change in color and characteristics of some samples were observed as shown in Table 1.

Table 1: Characteristics of human clinical urine and Pus samples

Sample	Color
Urine	Pale yellow
Urine	Yellow
Urine	White
Urine	Greenish
Pus	whitish yellow
Pus	Yellow
Pus	yellow brown
Pus	Greenish

Table 1: To study the percentage positivity of *P. aeruginosa* and its antibiotic susceptibility in hospitalized patients total 105 human clinical Samples were taken from different Patients of different wards (males and females) of the intensive care unit (ICU), urology ward, and OPD ward of different hospitals in Faisalabad.

Isolation and identification of *P. aeruginosa*

For the isolation of *P. aeruginosa,* samples were swabbed on the blood and MacConkey agar completely with the help of sterile swabs. After swabbing, plates were kept in the incubator for 24 hours at 37 °C. After incubation, plates were observed for beta hemolysis on blood agar and no agar color change on MacConkey agar. It showed green color due to the production of Pyocyanin pigment. Smooth, flat, and opaque growth with rotten grape-like smell was observed on plates.

Purification of *P. aeruginosa* from human clinical samples

Purification of *P. aeruginosa* was done for further confirmation and biochemical characteristics of isolates. After the incubation of 24 hours few cells were taken with the help of properly sterilized inoculating loop and streaked over cetrimide agar plate for the purification of *P. aeruginosa*. Cetrimide agar acts as selective medium for *P. aeruginosa* and due to the production of pyocyanin pigment, which easily diffuses into agar and gives green color.

Microscopic Examination

For the microscopic characterization of Gram's Staining was performed. After Gram's staining slides were examined under light microscope at 100x magnification. *P. aeruginosa* appeared as large rods. Rods were pink in color which indicates these are gram-negative rods.

Table 2: Gram's staining of *P. aeruginosa* isolated from clinical samples

Gram stain	Negative
Shape of bacteria	Rods
Color	Pink
Arrangement	Large Single Rods

Table 2: Gram's staining of *P. aeruginosa* isolated from clinical samples and after staining the slides were examined under microscope at 100x objective lens for the confirmation of *P. aeruginosa*.

Biochemical characterization of *P. aeruginosa*

For the confirmation and biochemical characterization of isolates, biochemical tests including methyl red test, Glucose fermentation test, catalase test, citrate and Oxidase Test were performed. For oxidase catalase, citrate and methyl red. *P. aeruginosa* tested positive for Oxidase, Citrate Catalase, and Sugar Fermentation tests.

Table 3: Biochemical Characterization of *P. aeruginosa*

Biochemical test	Result
Catalase Test	Positive
Glucose Fermentation Test	Positive
Citrate Utilization Test	Positive
Methyl Red Test	Negative
Oxidase Test	Positive

Table 3: Biochemical Characterization of *P. aeruginosa.* Biochemical test was performed for the phenotypic Characterization of *P. aeruginosa.*

Percentage positivity of *P. aeruginosa* isolated from human clinical samples

From total of 105 Samples 63 samples were Positive for *P. aeruginosa*. Out of 63 samples, 27 were positive for males while 36 were positive for *P. aeruginosa***.** The overall positivity percentage of *P. aeruginosa* was 60%. Samples were taken from patients of different age groups ranges from 16 to 80 years. Samples were taken from patients of different age groups ranges from 16 to 80 years. Patients with the age group of 40 60 years showed highest percentage positivity for *P. aeruginosa,* that is 71% while the range of 1 20 years showed lowest 27%

Table 4: Sample positivity in human clinical patients

Sample source	Sample positivity	Males	Females
Urology ward	42/65 (64%)	17/29 (58%)	25/36 (69%)
Emergency ICU	7/15 (46%)	4/9 (44%)	3/6 (50%)
OPD	14/25 (56%)	6/15 (40%)	8/10 (80%)
Overall percent positivity	63/105 (60%)	27/53 (51%)	36/52 (69%)

Table 4: The data was statistically analyzed by percentage positivity. *P. aeruginosa* isolated from human clinical samples and showed sample positivity. The overall positivity percentage of *P. aeruginosa* was 60%.

Antimicrobial Sensitivity Pattern for *P. aeruginosa*

The inhibition zone pattern was used to assess *P. aeruginosa* susceptibility to several type of antimicrobial drugs in Muller Hinton's agar using the conventional disc diffusion method. The Kirby Bauer technique was used to assess *P. aeruginosa* antibiotic sensitivity and resistance patterns. To identify isolates as susceptible, the zone diameter interpretive criteria for *P. aeruginosa* were employed. Results were determined by measuring diameter of zones around the discs according to Clinical and Laboratory Standards Institute (CLSI) guidelines, as shown in **Table 4**.

Table 5: Standard chart of *P. aeruginosa* CLSI guidelines 2020

Sr#	Antibiotics	Disc load	Resistance(mm)≤	Intermediate(mm)	Sensitive (mm) ≥
1	Meropenem	(l0µg)	15	16 18	19
2	Azithromycin	(15 µg)	13	14 17	18
3	Amikacin	(30 µg)	14	15 16	17
4	Gentamicin	(30 µg)	12	13 14	15
5	Aztreonam	(30 µg)	15	16 21	22
6	Enrofloxacin	(5 µg)	18	19 24	25

Table 5: The antipseudomonal antibiotics tested include Meropenem (l0µg), Azithromycin (15 µg), Amikacin (30 µg), Gentamicin (30 µg), Aztreonam (30 µg), and Enrofloxacin (5 µg) were impregnated over MHA agar with evenly spread culture of *P. aeruginosa* of composition 0.1ml. Plates were incubated for 24 hours at 37°C.

Biofilm Formation of *P. aeruginosa*

According to established techniques, microtiter plate-based biofilm quantification experiments were carried out employing adherent cell staining with crystal violet (CV). *P. aeruginosa* strains from overnight cultures were subcultured at 37°C in 96-well microtiter plates at an optical density (OD) OD600 of 0.2. Absorbance was measured at 550 nm using specific Thermo Multiskan fc reader. In 96-well microtiter plate 45 samples out of 63 positive samples were positive with a positivity rate of 71.43 percent. Most of the biofilm positive isolates had higher antibiotic resistance than the biofilm-negative isolates. Isolates had a maximum 46 percent resistance to azithromycin. The percentage positivity of biofilm-producing & non biofilm producing *P. aeruginosa* isolated from clinical samples was shown in Figure 1.

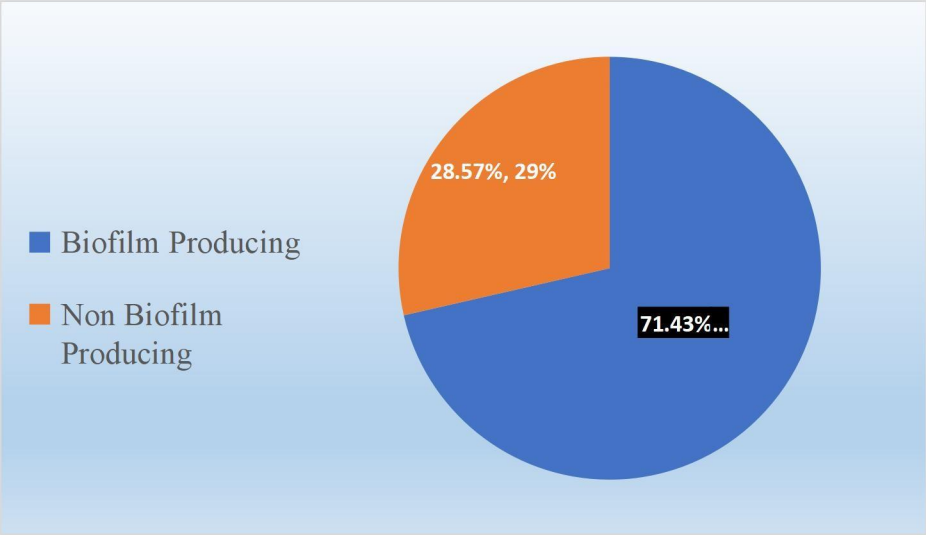

Figure 3: Percentage positivity of biofilm-producing & non biofilm producing *P. aeruginosa* isolated from clinical samples. Most of the biofilm-positive isolates had higher antibiotic resistance than the biofilm-negative isolates.

Discussion

A Gram-negative bacterium called *P. aeruginosa* can cause significant illnesses in persons with AIDS or cystic fibrosis. It can be found in a range of biological systems (Brindhadevi et al., 2020). This can cause biofilm to form on a number of surfaces, enabling the bacteria to generate defense mechanisms and increase their antimicrobial resistance, making them more resilient to harsh environments (Morin et al., 2021). Leading causes of contamination-related illness and mortality worldwide is *P. aeruginosa* obstruction (Zhang et al. 2020). *P. aeruginosa* is a significant hospital acquired bacterium that is a significant cause of death in chronically infected people with cystic fibrosis and produces a wide spectrum of acute infections (Reynolds and Kollef, 2021). In immunocompromised persons such as cancer patients who have consumed real food and those who have been infected with the human immunodeficiency virus *P. aeruginosa* produces serious diseases such irresistible

illnesses (Behera et al. 2018). *P. aeruginosa* was identified as among the most dangerous bacteria in 2017 by WHO and as a crucial germ for cutting-edge anti-toxin research (Abaza et al. 2017) (Grainha et al., 2020). *P. aeruginosa* has the ability to produce extremely complex biofilms which are frequently discovered in patients with chronic illnesses such persistent lung infection (Bisht et al., 2020). Both persistent rhinosinusitis and ongoing damage contamination might affect a person at the same time. In the present study, 105 samples were cultured out of which 63 were positive. Positive samples underwent additional processing to test the impact of biofilm on patterns of antibiotic sensitivity. The data show that *P. aeruginosa*'s antibiotic resistance is greatly impacted by biofilm. A study conducted in Egypt to check the occurrence of *P. aeruginosa* cultured from 100 clinical samples revealed that the overall percentage positivity of 100 samples was 63. Gentamicin, amikacin, imipenem, and ciprofloxacin have all been shown through data from numerous authors to be effective treatments for infections brought on by *P. aeruginosa* (Aneela et al. 2019). Our research revealed that carbapenems were the most effective antibiotics, with resistance rates for imipenem and meropenem of 15 and 204% respectively. The least effective drug, enrofloxacin has a 13% resistance rate. Identical to our findings a study conducted in Turkey found that imipenem and meropenem resistance rates from the carbapenem classes were 15 and 20 percent, respectively. Another study found that gentamicin showed the highest resistance among the aminoglycosides (51.92%) and that low resistance was seen with other aminoglycosides such as amikacin (29.8%) and tobramycin. Resistance to amikacin and azithromycin was more pronounced in our study. *P. aeruginosa* has the greatest fluoroquinolone resistance rates, with 20–35 percent resistance to ciprofloxacin and amikacin, respectively (Lila et al. 2018). Similar findings were observed for ciprofloxacin and enrofloxacin resistance rates which ranged from 12 to 28%. The majority of isolates in this study (47.5%) came from ICU patients and the respiratory specimens and wounds (65%) had the highest percentages of isolates (22.5 %). They discovered that carbapenems were the most effective antibiotics with resistance rates for imipenem and meropenem of 15.4 and 20.4% respectively. The least active agent was gentamicin (Chand et al. 2021) . In our research, we discovered that gentamicin lagged behind Meropenem in terms of activity. Because of regional differences or hygienic circumstances results differed. In our research we discovered that gentamicin lagged behind Meropenem in terms of activity. Because of regional differences or hygienic circumstances, results differed (Chatterjee et al. 2016). In our investigation we reported a 63 percent rate of clinical human samples that were positive which is comparable to non- clinical human samples. Given the substantial literature review that has been done above it can be said that *P. aeruginosa* resistance has been steadily rising over time in Pakistan and throughout the rest of the world (Farooq et al. 2019). The unusual structure of *P. aeruginosa* which has a big genome with 6.3 million base pairs and is thought to be the largest sequence of all bacteria may be the cause of the rise in resistance to this organism (Jacobs et al., 2003). This sequence's adaptability results in inherent resistance to antibiotics and it also has the maximum number of regulatory genes that can cause an efflux pump mutation. In order to assure the prevalence and pattern of antibiotic resistance of *P. aeruginosa* a total of 105 samples were gathered from the various wards (intensive care unit (ICU) OPD and urology ward) of different hospitals. The prevalence and antibiotic susceptibility of *P. aeruginosa* isolated from urine samples were examined in a similar investigation. Collected samples were prepared for *P. aeruginosa*

isolation on selective media such as blood MacConkey and cetrimide. Colonies ranging from green to yellow brown were found. Standard microbiological methods were used to characterize isolates. Biochemical testing confirmed *P. aeruginosa*'s presence (Abdulhaq et al., 2020). Isolates were stained using Gram's reagent, which revealed them to be pink rod-shaped pathogens. Numerous biochemical tests were carried out, including those for oxidase catalase citrate and methyl red. *P. aeruginosa* tested positive for Oxidase, Citrate Catalase, and Sugar Fermentation tests. On Muller Hinton agar antibiotic sensitivity was tested against a variety of medications, including Meropenem, Azithromycin Amikacin, Gentamicin, Aztreonam and Enrofloxacin. Maximum *P. aeruginosa* isolates tested positive for multiple antibiotic resistance patterns. 45 clinical samples out of 68 positive samples were MDR because of biofilm formation with a positivity rate of 71.43 percent. Results may slightly vary as a result of regional variances, hygienic circumstances, and antibiotic overuse (Jawad 2016).

P. aeruginosa worldwide accounts for 10–15 percent of infectious illnesses. Due of the species' innate tolerance to different antimicrobial drugs as well as its extraordinary ability to create new mechanisms of resistance, the majority of these infections are challenging to cure. An antibiotic-resistant organism known as *P. aeruginosa* demonstrates almost all recognized enzymatic and mutational pathways of bacterial resistance (Lister et al., 2009). One of the main mechanisms for species endurance when living conditions change quickly, such as temperature and food availability, is the biofilm which is an expansion of bacteria enclosed in a self-created matrix of extracellular polymeric molecules (EPS). The majority of *P. aeruginosa*'s biofilm matrix is made up of polysaccharides, proteins, lipids and extracellular DNA (eDNA) (Lai et al., 2022). About 90% of the biomass in biofilms is made up of the matrix which serves as a link between living and non-living surfaces and a haven for bacteria trapped in hazardous conditions (Castanheira et al., 2022). Antimicrobial resistance is steadily rising in clinical isolates. In order to learn more about the prevalence and profile of antibiotic resistance among clinical patients the current investigation was carried out (Cabot et al., 2016).

This study can be processed further for Geno typing and PCR to find out the most resistant strains of *Pseudomonas aeruginosa* that are strong biofilm forming. Moreover, we can find out techniques for battling bacterial biofilms and increase the use of anti-biofilm substances and their modes of action. We can also figure out about inhibition of AHL-mediated quorum sensing.

Conclusion

Our study showed that it is imperative that build up novel and effective treatments against *P. aeruginosa* as it is an infectious agent that resists multiple drugs and a main contributor of nosocomial catheter associated and UTI infections so it is becoming a serious health issue with the passage of time. Moreover, there must be a ban over misuse of antibiotics without the permission of certified physicians. A biofilm-forming opportunistic bacterium called *P. aeruginosa* causes chronic infections in immunocompromised patients and has a high fatality rate. Clinical isolates of *P. aeruginosa* that include unique epigenetic markers may produce excessive biofilms which could increase their antibiotic resistance and in vivo colonization. Higher antimicrobial resistance and decreased pathogenicity brought on by biofilm formation enable *P. aeruginosa* to persist in the host for an extended period of time.

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