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Molecular And Phenotypic Analysis Of Pseudomonas Aeruginosa Strains Isolated From Chronic Wound Infection

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ABSTRACT

After Escherichia coli and Staphylococcus aureus, P. aeruginosa is regarded as the third nosocomial pathogen. Additionally, it is the leading cause of hospital infections, particularly in patients who have burns, immunosuppression, or intubation due to cystic fibrosis (CF). In many parts of the world, P. aeruginosa resistance to antipseudomonal medications has been steadily rising over time. The aim of the current study is to isolate P. aeruginosa strains from chronic wound infections and analyze them on the basis of molecular and phenotypic characteristics. In the study total 100 samples were collected from burn unit patients with mild to severe burn wounds exhibiting burn infection symptoms from district Abbottabad. The isolated bacteria were then characterized on the basis of their morphology and microscopic analysis. All strains are classified and characterized as P. aeruginosa by giving pale colonies on MacConkey media. Biochemical characterization was conducted to characterize isolated pathogens. The biochemical test results show that isolates show negative results towards methyl red, and positive results towards catalase and oxidase. Resistance profiles were evaluated through the use of antibiotic susceptibility testing. DNA extraction was conducted using ladder of 1500 bp the results show P. aeruginosa band size is of 700bp. The phylogenetic analysis results confirm the isolated bacterial pathogen as P. aeruginosa strains.

INTRODUCTION

DOI: Availability

The gram-negative, aerobic, non-spore-forming rod *P. aeruginosa* might infect both immunocompetent and immunocompromised hosts with a range of diseases. It is a very difficult organism to treat in modern medicine because of its propensity to infect immunocompromised hosts, remarkable adaptability, resistance to antibiotics, and a variety of active defenses (Reynolds and Kollef, 2021). The environment frequently contains *P. aeruginosa*, especially in freshwater. Swimming pools, jacuzzis, and hot tubs are prominent instances of reservoirs in urban areas. *P. aeruginosa* causes a number of illnesses. Localized infections after burns or surgery may lead to widespread and often lethal bacteremia (Wu *et al.*, 2015).

In hospital setting *P. aeruginosa* gets introduced on catheters which results in urinary tract infections. Moreover, *P. aeruginosa* colonization is persistent in the majority of individuals with cystic fibrosis. *P. aeruginosa* bacteremia is uncommon in people with cystic fibrosis, which is likely due to high levels of *P. aeruginosa* antibodies in the blood (Krell and Matilla, 2024). The majority of people with cystic fibrosis, however, pass away from localized *P. aeruginosa* infections. By using infected respirators, additional patients may get necrotizing *P. aeruginosa* pneumonia. After eye surgery or trauma, serious corneal infections can be caused by *P. aeruginosa* (Bassetti *et al.*, 2018). A single polar flagellum allows almost all strains for movement, whereas others contain two or three flagella. Heat-labile antigens (H antigen) are produced by the flagella. Pili, which are typically present in clinical isolates, may have antiphagocytic properties and likely facilitate bacterial adhesion, which in turn promotes colonization (Chevalier *et al.*, 2017). Like other Gram-negative bacteria, *P. aeruginosa* has three layers in its cell envelope: the outer membrane, the peptidoglycan layer, and the inner or cytoplasmic membrane (Wiehlmann *et al.*, 2007). Phospholipid, protein, and lipopolysaccharide (LPS) make up the outer membrane. In addition to side-chain and core polysaccharides, the LPS of the majority of *P. aeruginosa* strains contains hydroxy fatty acids, 2-keto-3-deoxyoctonic acid, and heptose. On blood agar plates, practically every strain of *P. aeruginosa* is hemolytic, and a number of distinct hemolysins have been identified (Chevalier *et al.*, 2017).

P. aeruginosa is recognized to produce strong biofilms that are extremely resistant to host defenses, disinfectants, and antibiotics. This hinders bacterial removal and causes the development of extremely resistant chronic infections, which are a serious medical concern (Olivares *et al.*, 2020). Three exopolysaccharides (EPSs) the capsular polysaccharide alginate and two aggregative polysaccharides, Psl and Pel make up more than 50% of *P. aeruginosa*,

although it also includes proteins and extracellular DNA (eDNA). *P.aeruginosa* forms "Capped" mushroom-shaped structures and an intricate network of channels that disperse nutrients and oxygen while eliminating waste which represents the features of mature *P. aeruginosa* biofilms (Chatterjee *et al*., 2016). *P. aeruginosa* antibiotic resistance is still a major cause of morbidity and death and a severe health concern, particularly in intensive care units (ICUs) and long-term care hospitals (LTACHs). According to CDC data, the percentage of MDR *P. aeruginosa* isolates decreased from 15.7% in 2011 to 9% in 2018 (Chatterjee *et al*., 2016). The National Healthcare Safety Network's 2015–2017 data indicates that *P. aeruginosa* antibiotic resistance is still a major worry. 26.3%, 26.5%, and 27.1% of *P. aeruginosa* isolates from intensive care unit patients were resistant to carbapenems, extended-spectrum cephalosporins, and fluoroquinolones, respectively (Ibrahim *et al*., 2020).

Furthermore, isolates from intensive care unit patients were found to be MDR, indicating they were resistant to three or more antibiotics, in 18.6% of cases. Considering how frequently *P. aeruginosa* is resistant to other antimicrobials, carbapenem resistance in this bacteria can make treatment plans more difficult (Wüllner *et al*., 2022). Context-specific measures might increase the effectiveness of certain medications against *P. aeruginosa* infections. In fact, the combination of cephalosporin and beta-lactamase inhibitor ceftolozane/tazobactam (C/T) demonstrated both safety and effectiveness in treating highly drug-resistant *P. aeruginosa* in ventilator-associated pneumonia (Zelenitsky *et al*., 2003).

MATERIALS AND METHOD

In the Abbottabad district, this cross-sectional study was conducted. A total of 100 *P. aeruginosa* strains were obtained from burn unit patients with mild to severe burn wounds exhibiting burn infection symptoms.

INCLUSION CRITERIA

Patients whose wounds have not healed after at least four weeks. The median age of the patients was 5 years (range: 1–55 years). Among them, 54% were female and 46% were male.

SAMPLE COLLECTION

Using sterile cotton-tipped applicators, swab samples were collected from the wound surface and sent immediately to the lab in a cold chain setting.

IDENTIFICATION OF ISOLATED *P. AERUGINOSA*

The specimens were cultivated on 5% Blood agar and MacConkey agar plates, and they were

incubated for 24 hours at 37 °C in an aerobic environment. By 42ºC, well-isolated colonies had been identified as *P. aeruginosa*.

GRAM STAINING

To perform the Gram staining, a small amount of distilled water was applied to a transparent slide. A sterile needle was used to apply a little amount of pure culture on the slide. The culture was evenly distributed throughout the surface using the needle. The smear was formed using sterilized water, a drop of crystal violet was added to the slide smear. The crystal violet was swirled in and let to dry for around 30 seconds once a uniform dispersion was achieved. Following the application of crystal violet stain, the slide was carefully cleaned using sterile distilled water. The slide was properly washed with pure distilled water, and then a droplet of Lugol's iodine was applied to the smear to remove any leftover crystal violet hue. Lugol's iodine and crystal violet combine to keep the stain in place. After applying Lugol's iodine, the slide was cleaned with acetone. Acetone, a decolorizer, aids in removing excess stains from the slide. To cover up the smudge, a drop of safranin was put on the slide. The counterstained safranin stain gives gram-negative bacteria their distinctive color. After thoroughly cleaning the slide with water to get rid of any remaining traces of safranin, it was eroded clean. Blotting paper was used to gently remove the excess liquid from the slide. To observe the slide containing the plated smear, a 100X magnification microscope was utilized (Coico, 2006).

BIOCHEMICAL CHARACTERIZATION

Biochemical assays, including catalase, oxidase and methyl red test were carried out. Briefly described as follow:

CATALASE TEST

The test indicates the presence of the catalase enzyme. It is employed to distinguish between different bacteria that produce the catalase enzyme. The *P. aeruginosa* strain catalase test was carried out by gently mixing one colony with hydrogen peroxide on a sterile slide. The appearance of gas bubbles on the surface of the culture material indicated that the test was positive (Reiner, 2010).

OXIDASE TEST

The oxidase test is a method for determining if cytochrome C oxidase, also known as cytochrome a3, an enzyme involved in aerobic respiration, is present. A small piece of filter paper was treated with 1% Kovac's oxidase reagent and left to air dry. A well-isolated colony of *P. aeruginosa* strains a freshly cultured (18–24 hours) was taken from bacterial plate onto filter

paper using a sterile loop. Color differences were analyzed for each test colony. After an oxidase-positive test, the color turns dark purple in ten to fifteen seconds. The color either remains the same or reacts more slowly than two minutes when oxidase-negative organisms are present (Shields and Cathcart, 2010).

METHYL RED (MR) TEST

When the methyl red indicator is added at the end of the incubation time and changes color, it indicates that the fermentation of glucose produced enough acid and that the conditions were favorable to keep the pH of an old culture below 4.5. The Methyl Red (MR) Test was conducted by inoculating a culture of the *P. aeruginosa* strains into a tube filled with MR broth, which was rich in peptone and glucose. Test tubes were filled with two loopfuls of each bacterial culture, incubated for 48 to 72 hours at 37°C, and labeled with the name of the organism. Following the duration of incubation, a few drops of methyl red indicator were added to the incubated tubes. Every tube was analyzed to check for a certain color (Tille and Bailey, 2014).

INDOLE TEST

The indole test is used to determine if an organism can convert tryptophan into indole. Indole was identified using the Kovac's reagent, which mixes concentrated hydrochloric acid, isoamyl alcohol, and paradimethylaminobenzaldehyde in an acidic environment. A culture of *P. aeruginosa* was added into a tube filled with tryptophan broth and incubated at 37 °C for 24 to 48 hours in order to conduct the Indole test. Add 0.5 ml (5 drops) of Kovac's reagent and mix gently. Observe at the topmost layer of the liquid; if there are purple or red rings visible, a favorable result is shown; if yellow rings appear, a negative outcome is shown (KOMAL, 2019).

MOTILITY TEST

This test is done to find out if an organism can move using its flagella. The placement of the flagella differs depending on the type of bacterium. In order to perform the motility test for *P. aeruginosa* strains transfer the semisolid agar into test tubes after preparing it. Apply a straight needle to a colony of a culture that has grown on nutrient agar medium for 18 to 24 hours. Once at the middle of the tube, only pierce 1/3 to ½ inch deep. Make sure the needle exits the medium in the same direction as it entered. Incubate for up to seven days at 35°–37°C to see if a diffuse growth zone has flared out from the inoculation line (Shields and Cathcart, 2011).

MOLECULAR CHARACTERIZATION

DNA EXTRACTION

For DNA Extraction, colonies were grown on MacConkey media for 48 hours at 28 °C, and genomic DNA were extracted using a boiling method. A loop full of *P. aeruginosa* strains was picked up and suspended in TE buffer [100 mM Tris (pH 8.0), 30 mMmg/ml, 20 µl) was added into the tube and incubated at 37°C for 30 min. SDS [10% (w/v), 40 µl] was added, and the tube was inverted for 5-6 times, then incubated at 55 °C for 10 min. Phenol: chloroform isoamyalcohol [400 µl] was added and mixed well. The mixture was spun at 13,000 rpm for 15 min. The upper solution was transferred to new microtube. The same procedure was repeated if the solution was not clear. 3 M Sodium acetate (1/10 volume) and 2 volume of cold ethanol was then added. DNA was then pooled by glass rod and dried for 5-10 min until DNA became clear. Finally, the DNA was dissolved in 100 - 200 µl of sterilized distilled water and stored at - 20°C (Izadiyan and Taghavi, 2020).

PCR

PCR was carried out to molecularly confirmed the pathogen using specific primers. PCR was carried out by preparing the reaction mixture. The PCR master mix contained 1 µMol l−1 of each primer, 0.2 mMol l−1 dNTPs, and Taq buffer (67 mM l−1 Tris HCl pH 8.8). 5 U (1 µl) of Taq DNA polymerase added to the PCR tube to prolong enzyme's working life after the initial (one-time) denaturation phase, which was carried out at 95 degrees centigrade for 5 minutes. For the purpose of amplifying DNA, the following temperatures were used: 95 °C for five minutes (once), 94 °C for thirty seconds, 47 °C for thirty seconds, and 72 °C for fifty seconds. Following twenty cycles, the process was allowed to complete extension at 72°C for eight minutes (Ståhlberg *et al.*, 2012).

GEL ELECTROPHORESIS

2% agarose gel was prepared by dissolving 0.66 g in 30 ml TBE buffer, heated in an oven for 2 min. Ethidium bromide (0.5µg/ml) was added to the gel and poured into gel tray when it temperature was about 45-50°C and allowed for solidification. The gel was completely submerged in TBE buffer. The PCR products was added by 2 µl blue tracking dye and loaded separately into the wells of the gel. 1 kb DNA ladder was also used as marker for comparison. A negative control was also included (master mix only and extracted DNA). Electrophoresis was performed at 200 volts for 30 mints. Gel was observed under UV light and photographed using UV-tech machine (Izadiyan and Taghavi, 2020).

RESULTS

COLLECTION AND TRANSPORTATION OF CLINICAL WOUND SWABS

A total of 100 wound swabs were obtained from the patients. Pus discharge from the wounds was aseptically collected using a sterile cotton swab, which subsequently added in sterile Brain Heart Infusion (BHI) broth in a universal container. The results of the current investigation indicated that 36 (36%) out of 100 specimens tested positive for *P. aeruginosa* infections, whereas 64 (64%) out of 100 specimens tested negative for *P. aeruginosa* infections. The patient cohort comprises 51 men and 49 females, categorized into three distinct age groups: 1-25, 26-50, and 51-75 years. The distribution findings of *Pseudomonas aeruginosa* isolates indicated that *P. aeruginosa* is related with infections in various individuals (Figure 1 and 2).

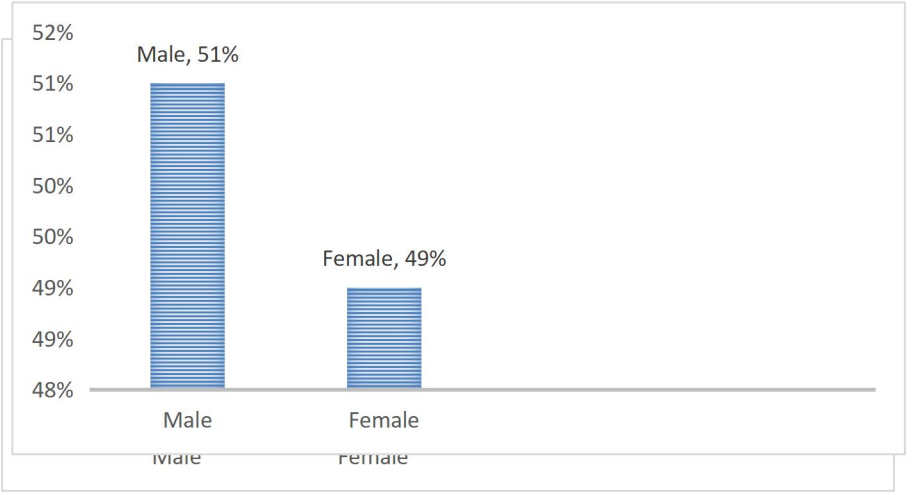


FIGURE: 1 PERCENTAGE OF MALES AND FEMALES OUT OF TOTAL 100 PATIENTS

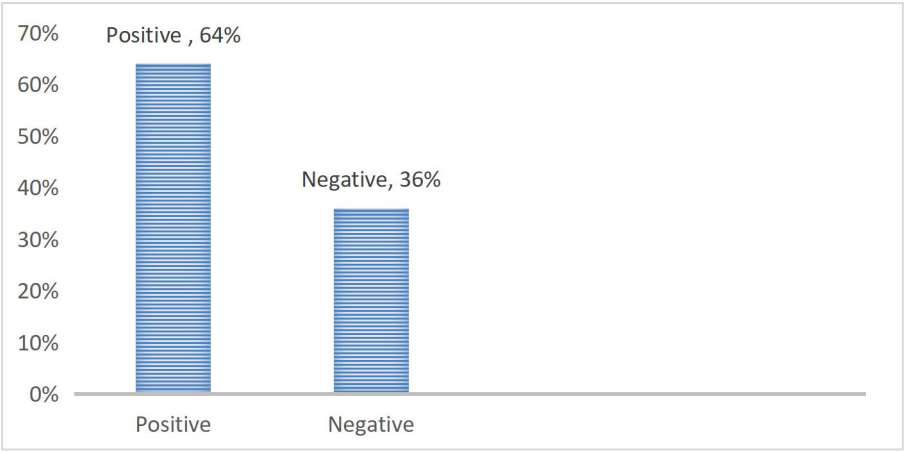


FIGURE: 1 PERCENTAGE OF POSITIVE AND NEGATIVE OUT OF TOTAL 100 PATIENTS

IDENTIFICATION OF BACTERIAL ISOLATES

Samples were pre-enriched in buffered peptone water and incubated at 37°C for 18–24 hours.

Enrichment was performed using nutrient broth. Culturing was done on nutrient agar, where metallic green colonies show typical *P. aeruginosa* morphology. Out of 100 samples 64 were *P. aeruginosa* pathogen isolated from chronic wound as PA1, PA2, PA3……..PA64 (figure 3).

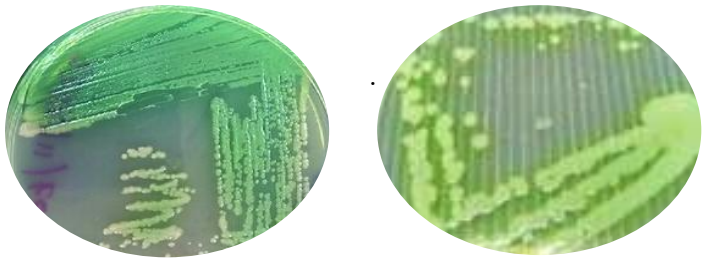


FIGURE: 3 MORPHOLOGY OF ISOLATED P. AERUGINOSA ISOLATE

GRAM STAINING

Using an isolated strain of *P. aeruginosa* cultured for a whole night, Gram staining identified the organism are Gram-negative rod shaped bacteria (Figure 4).

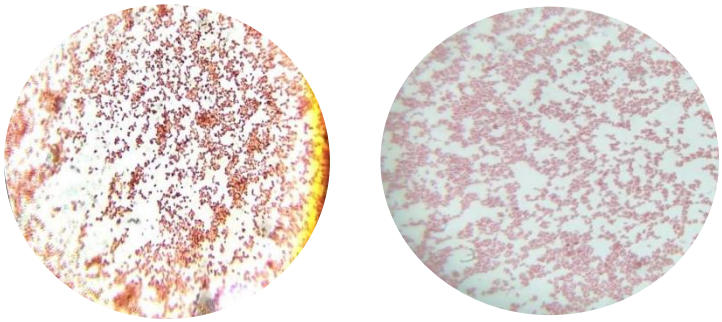


FIGURE: 4 GRAM-NEGATIVE ROD SHAPED BACTERIA UNDER MICROSCOPY

CATALASE TEST RESULTS

The isolated bacteria generated gas bubbles on a glass slide after being treated with a few drops of 3% H_2O_2, indicating that the catalase test was positive. The catalase test result showed that all the *P. aeruginosa* bacterial strain were positive (Figure 5).

FIGURE: 5 CATALASE TEST OF ALL BACTERIAL ISOLATE

OXIDASE TEST RESULTS: Kovac's oxidase reagent was used for this purpose. The ability of the organism to synthesize the cytochrome c oxidase was assessed by using the oxidase test.

The bacterium was positive to test whether the purple colour formed between 30-60 seconds. Our study's isolates *P. aeruginosa* were all oxidase positive.

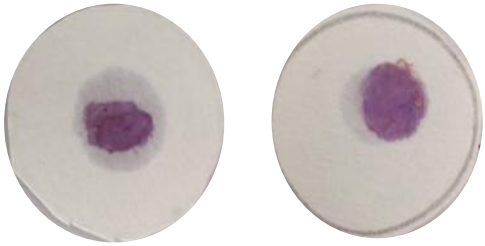
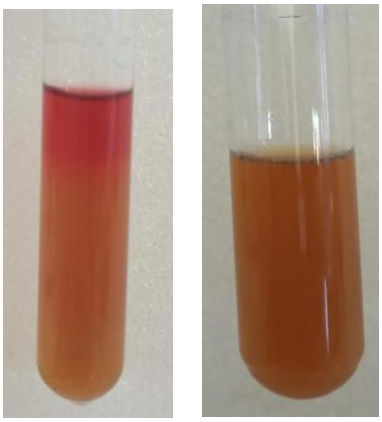


FIGURE: 6 OXIDASE TEST OF ALL BACTERIAL ISOLATES

METHYL RED (MR) TEST RESULTS

This test was conducted using glucose phosphate broth. This was done using methyl red indicator. An aliquot of culture broth containing methyl red indicator will become red when it is introduced, indicating a favorable outcome and the ability of the bacteria to consume glucose to create stable acid. The color of the methyl red indicator will shift from yellow to red. The outcome of the Methyl red test for the bacterial isolates of *P. aeruginosa* were negative (Figure 7).



A **B**

FIGURE: 7 METHYL RED TEST FOR ALL BACTERIAL ISOLATES, A USED AS A POSITIVE CONTROL AND B SHOW NEGATIVE RESULTS FOR ALL ISOLATES

MOTILITY TEST

Bacterial motility was assessed in this assay using a semisolid agar substrate. Bacterial motility is shown by a diffusive zone of growth from the inoculation line. *P. aeruginosa* strains all show

motility by spreading from the inoculation line.

FIGURE: 8 MOTILITY TEST FOR ALL BACTERIAL ISOLATES

INDOLE TEST

A reddish-colored ring appeared on the glass tube surface as soon as the kovac's reaction was added, indicating a successful indole test. When indole negativity is present, it is yellow or absent. Upon adding five to six drops of Kovac's reagent, every bacterial isolate was negative towards indole test (Figurer 9).

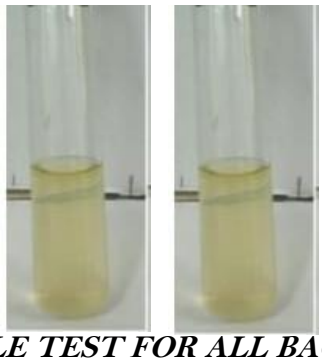


FIGURE: 9 INDOLE TEST FOR ALL BACTERIAL ISOLATES

DNA EXTRACTION

The GJC DNA Purification kit was used to extract the DNA from the sample at 20 degrees. The produced DNA was preserved and utilized as a PCR template. The extracted DNA served as a template for PCR testing and was stored at 20°C.

Stage	PCR Protocol	Temperature (°C)	Time (min.)
1st	Initial Denaturation	94	5.0
2nd (35 Cycles)	Denaturing	94	0.5
	Annealing	52.7	0.5
	Extension	72	2.0
3rd	Final Extension	72	5.0
4th	Hold	4	∞

PCR

The conserved region of test isolate was amplified by PCR. To successfully identify *P. aeruginosa*, a PCR approach was employed. The results show *P. aeruginosa* band size is of 700bp (Figure 10).

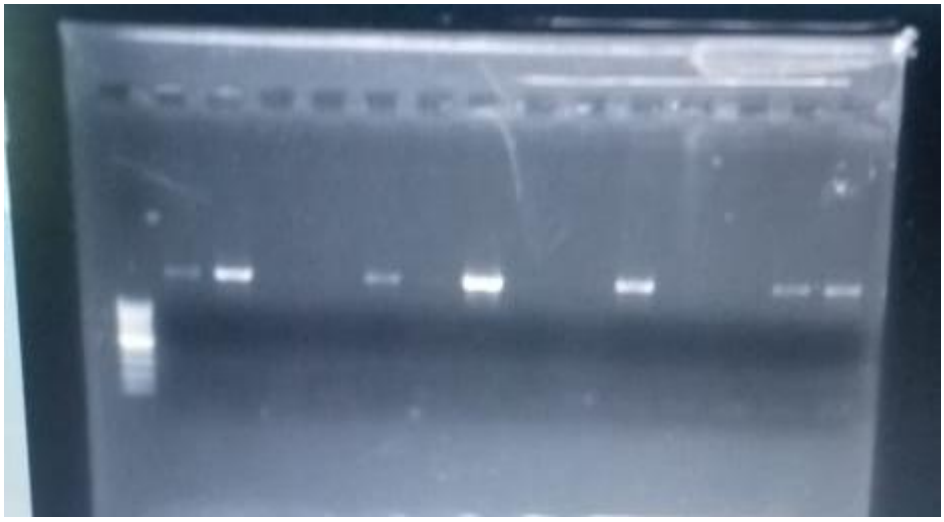


FIGURE 10: 16S RNA AMPLIFICATION RESULTS FOR ALL BACTERIAL ISOLATES

PHYLOGENETIC ANALYSIS

The ITS sequences of each bacterial isolate were Blasted using NCBI Nucleotide Blast, and the phylogenetic tree was built as described above. Every bacterial strain found during this inquiry has been identified to belong to the appropriate *P. aeruginosa* strains by the phylogram topology (Figure 11).

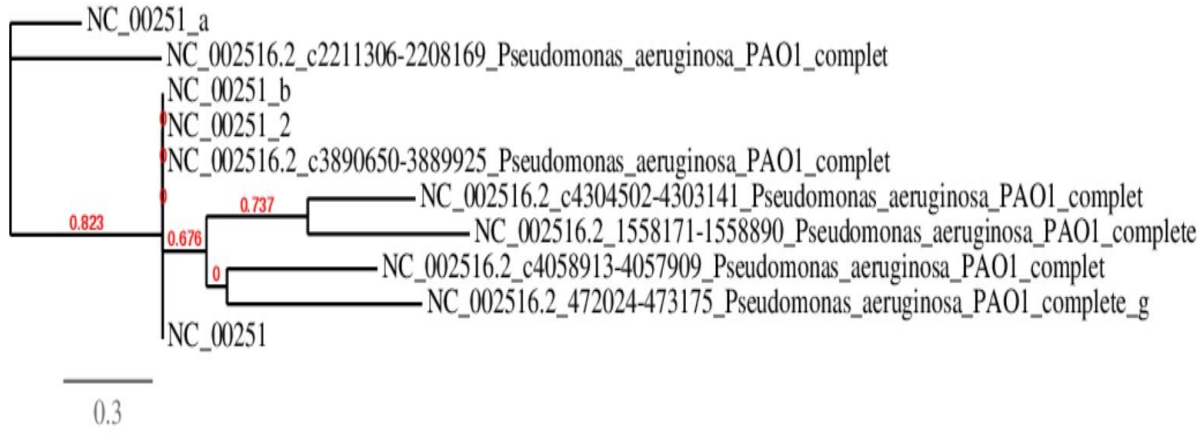


FIGURE 11. THE PHYLOGENETIC TREE OF ALL BACTERIAL ISOLATES

DISCUSSION

The gram-negative, aerobic, non-spore-forming rod *Pseudomonas aeruginosa* can cause a variety of illnesses in both immunocompetent and impaired individuals. Due to its severe flexibility, wide range of complicated defenses, antibiotic resistance, and aim to cause infections in immunocompromised hosts, it is a very challenging organism to treat in modern medicine. The development of numerous arrays of cell-associated and secreted virulence factors, such as structural elements, toxins, pigments, and enzymes (elastases, alkaline protease, pyocyanine, and rhamnolipids), which exacerbate the infection and disease process, is one of the reasons *P. aeruginosa* is regarded as an effective opportunistic pathogen. All of the isolates in our study had at least one distinct virulence characteristic (Chevalier *et al*., 2017).

Pseudomonas aeruginosa stains obtained from chronic wound infections and the aim of the current study's is to characterize the isolates on the basis phenotypic and molecular characteristics. One hundred *Pseudomonas Aureginosa* samples were taken from the Abbottabad district's burned unit. According to the current investigation's findings, 64 (64%) of 100 specimens tested negative for *P. aeruginosa* infections, whereas 36 (36%) of 100 specimens tested positive. A similar study by Ahmed *et al*., (2020) demonstrates that 150 samples from burnt units with persistent wounds were gathered. Fifty samples tested negative, while 100 samples out of 150 tested positive. MacConkey agar and blood agar were then used to describe the morphology of the bacterial isolates. *P. aeruginosa* strains form flat, pale, irregular colonies on MacConkey agar. Similar study was conducted by Badri (2019) which shows *P. aeruginosa* produce pale color colonies on the surface of MacConkey media.

An additional component of this investigation was the biochemical analysis of *P. aeruginosa*. Methyl red, oxidase, and catalase tests were performed in the current investigation. The experiment's findings demonstrated that the *P. aeruginosa* isolates tested negative for oxidase and positive for catalase in the methyl red test. Similar study was conducted by Ali *et al*., (2009) which shows *P. aureginosa* react to catalase and oxidase test while donot react with metyl red. Every isolate (100%) tested positive for the development of biofilms, confirming the phenotypic characterization of the virulence determinants by biofilm production. Of the isolates, 17 (42.5%) formed strong biofilms, while 14 (35%) produced moderate biofilms. Additionally, inadequate biofilm development was observed in nine (22.5%) isolates. The isolates that formed biofilms had higher levels of antibiotic resistance. Similar study was conducted by Badri (2019) which demonstrates that the formation of biofilms has been identified as a key factor in determining

the pathogenicity of *P. aeruginosa* infections. According to the microtiter plate test, the majority of them (84%) produced biofilms.

The purification kit for GJC DNA was used. The extracted DNA was used as a template for PCR testing and stored at 20°C. A PCR method was used to effectively identify *P. aeruginosa*. PCR is considered to be a less time-consuming method for the rapid and economical identification and detection of pathogenic microorganisms. Bacterial isolates are confirmed to be *P. aeruginosa* strains by the phylogram topology. Similar study by Abdeen (2017) which showed that the isolated strain exhibited a great deal of similarities with Pseudomonas species.

CONCLUSION

Based on the current investigation, we conclude that a substantial amount of *P. aeruginosa* burn wound isolates possessed a profusion of virulence enzymes, biofilm, and toxin production in addition to antibiotic resistance. These results highlight the necessity of focused antimicrobial treatments and accurate diagnostic techniques in the treatment of persistent wound infections caused by *P. aeruginosa*. Understanding strain diversity, resistance development, and any epidemic hazards related to this infectious agent requires ongoing surveillance and genetic analysis.

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