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SCREENING OF VIRULENCE FACTORS AMONG MULTIDRUG-RESISTANT ISOLATES OF PSEUDOMONAS AERUGINOSA FROM BURN PATIENTS

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Abstract

Pseudomonas aeruginosa is a Gram-negative micro-organism and one of the most isolated opportunistic nosocomial pathogens in burn patients. The rise of multi-drug resistant strains globally has significantly limited therapeutic options. This study explores some virulence factors of P. aeruginosa that are related to infection development in burn patients. A total of 63 swab samples were collected from burn patients who were admitted to Burns and Plastic Surgery Center Hayatabad, Peshawar through December 2023 to February 2024. Of these, 52 (82.5%) swab samples showed positive bacterial growth. Identification through morphological and biochemical tests were performed which showed that 15 isolates (28.8%) were colonized with P. aeruginosa. The result of antibiotic susceptibility testing through Kirby-Bauer agar disc diffusion method showed that high sensitivity was found for colistin (93.3%) and polymyxins-B (80%). Significant resistance rates included tazobactam (40%), salbactum (53.3%), aminoglycosides (53.3%), imipenem (40%), meropenem (46.7%), doripenem (66.7%), and ciprofloxacin and levofloxacin (46.7%). In addition, 46.67% isolates were found XDR and 26.67% were MDR isolates of P. aeruginosa. Virulence factors analysis showed that 12 isolates (80%) produced alkaline protease,9 isolates (60%) produced Lipase, and 13 isolates (86.67%) produced phospholipase. The substantial production of several phenotypic virulence factors underscores their critical role in infection spread, pathogenicity, and antibiotic resistance. Consequently, identifying anti-virulence agents for adjuvant therapy is vital in treating P. aeruginosa, particularly in multidrug-resistant (MDR) strains. This study emphasizes the need for continued research

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into alternative therapeutic strategies to reduce these challenging infections in burn patients.

INTRODUCTION

In 1882, Carle Gessard used his book "On the Blue and Green Coloration of Bandages" to describe how P. aeruginosa grew from cutaneous wounds of two patients who had bluish-green pus. This was the first successful isolation of the bacteria in pure culture. (Gessard, 1984) P. aeruginosa is a rod-shaped, asporogenous, monoflagellated, gram-negative bacteria that has a grape- or tortilla-like odour and a pearlescent look. P. aeruginosa develops best in temperatures between 25 and 37°C. Its capacity to grow at 42°C sets it apart from many other Pseudomonas species. It is the cause of disease in humans as well as in plants and animals. In patients with impaired immune systems, those with severe burns, and people with cystic fibrosis, it can lead to deadly infections (Jin, 2024) . This bacterium has a great deal of metabolic flexibility and regulatory genes that allow it to mature under a diversity of environments. It is extremely challenging to remove this bacterium from sick people due to its high antibiotic resistance, several virulence factors, and adaptability to different diets. This is specifically confirmed when it comes to lung infections in cystic fibrosis patients (Wu et al., 2015). A growing percentage of infections committed in the contemporary hospital context are caused by P. aeruginosa. With an attack rate of 36 infections per 10,000 hospital discharges, it represents 8.5% of all nosocomial infections. When it comes to nosocomial pneumonia and burn wound infections, P. aeruginosa is the most commonly isolated, opportunistic and multi-drug resistance (MDR) pathogens in patients (Morrison Jr and Wenzel, 1984). Hospitalized patients with diabetes, cancer, cardiovascular illness, or those using mechanical respirators are intensely susceptible to P. aeruginosa related pneumonia or bacteremia. Hospitalized patients who contract P. aeruginosa infections experience serious morbidity and death, as well as an absolute length of stay and higher expenses (Moore and Flaws, 2011).

1.2 General Characteristics of P. aeruginosa

1.2.1 Size and Shape

The rod-shaped, heterotrophic, motile, Gram-negative bacteria P. aeruginosa measures about 0.5–1.0 µm in width and 1-2 µm in length. It is a facultative aerobe that uses nitrate as the terminal electron acceptor to develop through both anaerobic and aerobic respiration. P. aeruginosa has controlled fermentative capabilities that typically tolerate very sluggish or no growth, and it can also increase anaerobically when given arginine (Diggle and Whiteley, 2020).

1.2.3 Pigmentation of P. aeruginosa

P. aeruginosa produces various pigments that contribute to its pathogenicity. Pyocyanin, a blue green phenazine, encourages oxidative stress and cellular injury through reactive oxygen species. Pyoverdine, a yellow-green, fluorescent siderophore-mediated pigment, is critical for iron sequestration, facilitating pathogen proliferation and virulence. Some strains produce additional pigments like pyorubin and pyomelanin, increasing the bacterium's chromatic spectrum. The biosynthesis of these pigments serves as an significant phenotypic trait for the clinical diagnosis of P. aeruginosa infections, providing insights into its pathogenesis and virulence mechanisms in burn patients (Abdelaziz et al., 2023).

1.3 Infection of P. aeruginosa

Over the last two decades, P. aeruginosa has become a significant pathogen. It is sensible for 10% to 20% of nosocomial infections. Acute leukemia, burn wounds, cystic fibrosis, organ transplant recipients, and intravenous drug addiction are among the situations that are most vulnerable to pseudomonas infection. Nosocomial contaminations, such as P. aeruginosa, are often found in hospital environments, and several objects have been associated with epidemics. Prolonged hospital stays are known to harbour this bacterium, which puts patients at higher risk of infection. Benign external otitis, endocarditis, pneumonia, meningitis, and septicemia are among the most serious infections (Bodey et al., 1983). P. aeruginosa was thought to be primarily a cause of strong antibiotic compounds, even though its pathogenicity was suspected and its capability to cause infections was known by 1889 (Botzenhart and Döring, 1993). Most pseudomonas infections show

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both invasiveness and toxicity. There are three distinct stages that make to the final Pseudomonas infection: first is colonization and adhesion of germs; second invasion of the local area; and third spread of systemic illness. But the illness process can halt at any point. Numerous virulence factors, such as those linked to cells and those released by the organism, contribute to P. aeruginosa multifactorial pathogenicity (Mohammad and Research, 2013).

1.3.1 Infection of P. aeruginosa in burn patients

Pseudomonas, particularly P. aeruginosa, is the most significant, resistant, and dangerous bacteria in burn patient infections. Infection is one of the most catastrophic effects for burn patients. In immunocompromised patients who have suffered thermal injuries, P. aeruginosa is extremely virulent and grows on the moist burn wound surface. Burn patients usually encounter these bacteria through burn wound cross-contamination. Pseudomonas infection is a frequent side effect that increases the morbidity and death rate of burn victims. The diagnosis is still bad, with a mortality rate of roughly 80% in such individuals, despite progresses in medical and surgical care (Lari et al., 2005).

1.3.2 Infection of P. aeruginosa in burn patients in Pakistan

In Pakistan, burn injuries are a common cause of morbidity and mortality. Burn injuries have a harmful effect on the immune system, making patients more susceptible to infection. The most noticeable result is injury to the skin, where the natural barrier is damaged and replaced by a vascular, protein-rich environment that creates an ideal setting for the growth of microorganisms. The primary cause of morbidity and death in burn patients is these infections. Gram-negative bacteria are the most common pathogens in burn wounds, with P. aeruginosa being the most common bacterium, according to a comprehensive analysis of burn wound infections (Saaiq et al., 2015). Over the course of two years, a study was conducted at the Burn Care Centre at the Pakistan Institute of Medical Sciences (PIMS), located in Islamabad, Pakistan. All patients, regardless of gender or age, who had a positive wound culture were involved in the study. P. aeruginosa accounted for 36 (35.29%) of the 95 positive microbial growths, making it the most instantly discovered isolate. In these patient cases, P. aeruginosa was the most widespread type of bacterial germ found in burn sites (Folkesson et al., 2012).

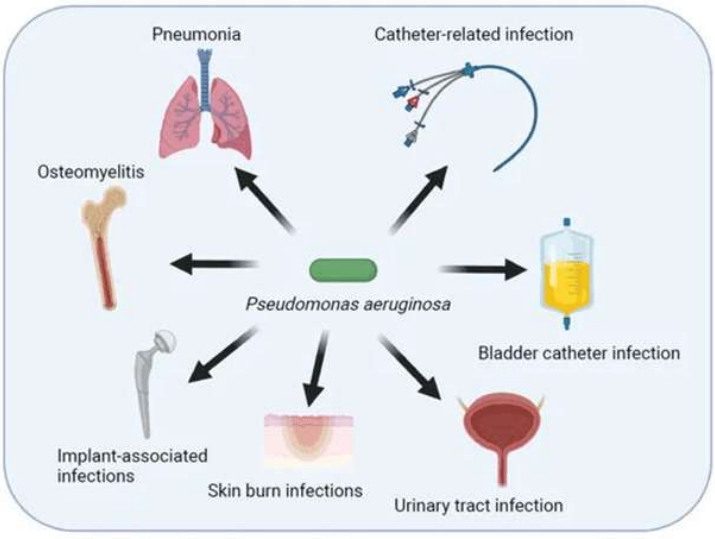

Figure 1.1 Schematic representation of main infections caused by P. aeruginosa (Tuon et al., 2022).

1.4 Virulence Factor

Targeting the extracellular matrix, several virulence factors can induce pathogenicity that promotes adhesion and impedes host cell signaling pathways. P. aeruginosa can invade an organism and cause several diseases that impair its immune system and make infections nearly impossible to treat (Zumla, 2010).Lipopolysaccharide, Flagellum, Type IV Pili,

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Type III Secretion System, Exotoxin A, Proteases, Alginate, Quorum Sensing, Biofilm Formation, Type VI Secretion Systems, and Oxidant Generation in the Airspace are a few examples of the pathogenicity brought on by virulence factors. Most of the P. aeruginosa outer membrane is made up of LPS. Bacterial lipopolysaccharide (LPS) is mostly composed of a hydrophobic domain called lipid A, also referred to as endotoxin. LPS is a major trigger for the host's innate and adaptive immune responses (Döring and Pier, 2008). Type IV pili of P. aeruginosa have a function in adherence to various cell types, which is probably significant in phenomena like attachment to specific tissues and tissue tropism. Phagocyte receptors that identify matching adhesions on microbial surfaces mediate the start of biofilm development (Frank, 1997). Type III Secretion System is a highly intriguing virulence factor that involves a flagellum-basal-body related system that delivers proteins directly from P. aeruginosa cytoplasm into the cytosol of host cells. This system helps P. aeruginosa evade phagocytosis and causes damage to host tissues, which in turn promotes immune avoidance and bacterial dissemination.(Passador and Iglewski, 1994) The elements of virulence Proteins such as lipase, phospholipase, alkaline phosphatase, and protease are secreted into the extracellular environment by the Type II secretion mechanism, which releases exotoxin A.(Pang et al., 2019)

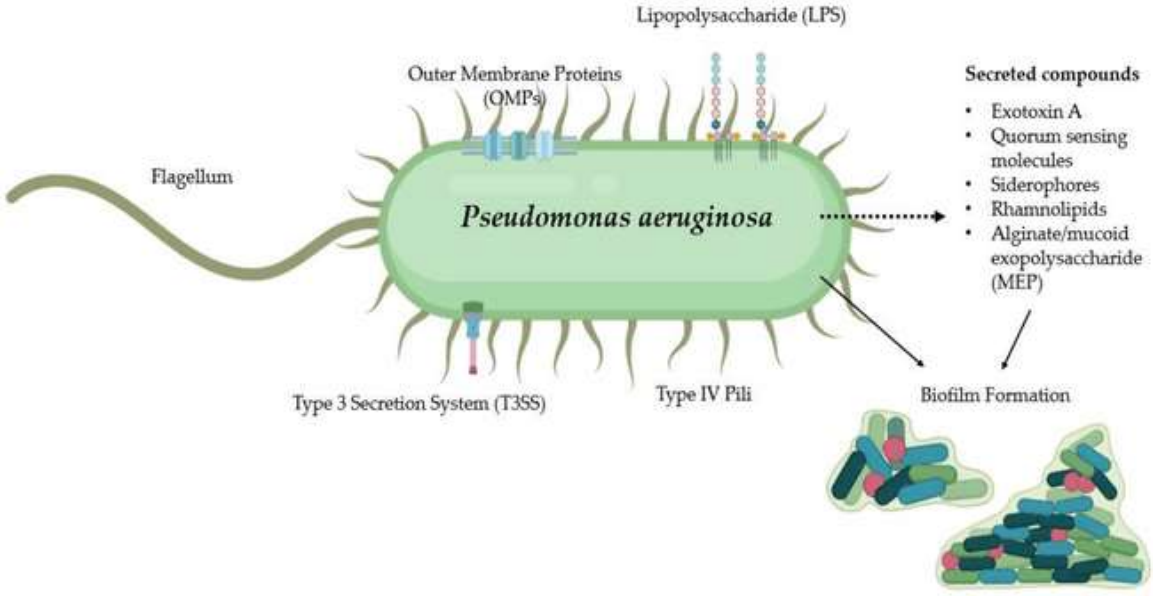

Figure 1.2 Key virulence factors important in the pathogenesis of P. aeruginosa infections (Killough et al., 2022).

1.5 Antibiotic resistance in P. aeruginosa

P. aeruginosa exhibits resistance to several antibiotics, such as β-lactams, aminoglycosides, and quinolones. The three main defense mechanisms P. aeruginosa uses against antibiotic attack are intrinsic, acquired, and adaptive resistance. Low outer membrane permeability, the development of efflux pumps that drive antibiotics out of the cell, and the synthesis of enzymes that render antibiotics inactive are all components of P. aeruginosa intrinsic resistance. P. aeruginosa can develop resistance by mutational alterations or horizontal transfer of resistance genes. P. aeruginosa develops an adaptive resistance through the production of biofilm in the lungs of infected individuals, which acts as a diffusion barrier to prevent antibiotics from reaching the bacterial cells (Breidenstein et al., 2011).

1.6 Multidrug Resistance (MDR) P. aeruginosa

P. aeruginosa is an important pathogen commonly implicated in nosocomial infections. The occurrence of multidrug-resistant (MDR) P. aeruginosa strains is

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increasing worldwide and limiting our therapeutic options. Rising antibiotic resistance increases the risk of multidrug-resistant infections in burn patients. Implementing effective antibiotic therapy protocols is crucial to combat MDR organisms and improve patient outcomes. Antibiotic resistance in P. aeruginosa is an increasing concern, characterized by the accumulation of resistance following exposure to various antibiotics and the development of MDR strains. This phenomenon has been particularly observed in patients with cystic fibrosis, where persistent P. aeruginosa infections lead to the sequential emergence of resistance to multiple antibiotic agents (Aloush et al., 2006). In the context of MDR P. aeruginosa isolates, it is widely acknowledged that reduced virulence may occur because of decreased bio fitness. This reduction in virulence is often associated with the acquisition of resistance mechanisms. Consequently, first-line agents used alone against MDR P. aeruginosa isolates are frequently ineffective. As a result, alternative or less preferred agents, which may have been previously overlooked due to concerns regarding their toxicity, are often required for treatment (Tam et al., 2010).

1.7 Extensively Drug Resistance (XDR) P. Aeruginosa XDR P. aeruginosa is strain that is resistant to at least one agent from all but two or fewer antimicrobial groups. XDR isolates are resistant to a wide range of antibiotics while remaining susceptible to only one or two classes of antimicrobial drugs. Some strains have developed resistance to several antibiotics, which could be mediated by several methods such as development of hydrolyzing enzyme, loss of outer membrane protein, efflux systems, and target mutations. This high level of resistance can substantially limit treatment options and is related with greater morbidity and mortality due to the difficulties of adequately treating these bacteria-caused diseases (Saderi and Owlia, 2015).

1.8 Background and gap analysis/problem statement

P. aeruginosa is a common and dangerous infection in burn patients. It is resistant to many antibiotics and possesses many virulence factors to attack host tissue. Further studies must be done to compare the actual outcomes of data in certain areas, to evaluate demographic risk factors, and to understand how virulence factors function to more completely judge and anticipate these diseases. Moreover, finding advantageous methods to treat as an anti-virulence agent is important to the issue of antibiotic resistance. Closing these gaps will aid efforts to comprehensively understand P. aeruginosa infections and ultimately minimize their public health burden.

OBJECTIVES

1. To isolate and identify P. aeruginosa strains from burn patients.
2. To determine the multidrug resistance profile of P. aeruginosa isolates.
3. To screen alkaline protease, lipase and phospholipase in MDR isolates of P. aeruginosa.

MATERIAL AND METHODS

2.2 Collection of samples

A total of 63 swab samples were collected from burn patients in Burns and Plastic Surgery Center Hayatabad, Peshawar during the period between December 2023 to February 2024 by sterilized cotton swabs soaked with NaCl. The patients who were included were within the age range of 2–75 years whose 20–50% body surface area (BSA) was involved in burns. The swabs were then transported to the Microbiological Laboratory of the Department of Microbiology, Hazara University Mansehra.

2.4 Sample processing

The samples were cultured on MacConkey's Agar, and Blood Agar and the plates were incubated at 35–37°C for 24–48 hours and examined for bacterial growth. Then, a colony was isolated and transferred to the liquid culture followed by a colony cultured in both blood agar and MacConkey agar for 24 hours at 37°C. The colonies were moved to the nutrient agar when they had culture characteristics resembling those of the bacteria being studied. After that, they were incubated for 24 hours at 37°C to make sure the isolates returned to the P. aeruginosa bacteria.

2.5 Identification of P. aeruginosa

P. aeruginosa identified through techniques such as Gram staining, culture characteristics, biochemical tests, and antibiotic susceptibility testing. P. aeruginosa colonies were identified by their pigment production, grape-like fragrance, oxidase positivity, motility, gram staining (as gram negative bacilli), non-

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fermentative nature, and growth at 42°C (Biswal et al., 2014).

2.6 Biochemical Tests

2.6.1. Gram Staining

The Gram stain distinguishes between Gram-positive and Gram-negative bacteria. Gram positive bacteria maintain the crystal violet stain, which appears purple, whereas Gram-negative bacteria lose the stain and appear pink when counterstained with safranin. Prepare a bacterial smear on a slide and heat-fix. Apply crystal violet, rinse, then add Gram's iodine. Decolorize with ethanol, then rinse and counterstain with safranin. Examine using oil immersion microscopy (Coico, 2006).

2.6.2. Catalase test

P. aeruginosa colony is transferred to a slide or test tube, and a drop of 3% hydrogen peroxide(H_2O_2) is applied. If bubbles or oxygen gas are detected, this demonstrates a positive catalase reaction, which is consistent with P. aeruginosa. The catalase test helps accurately identify gram negative bacteria (Reiner, 2010).

2.6.3. Oxidase Test

A combination of one percent aqueous dimethyl-p-phenylene-diamine and one percent α-naphthol in 95% ethanol is used for oxidase test. MacConkey agar colonies were covered with this mixture. In a matter of seconds, colonies that were positive for oxidase turned dark blue (Phillips, 1969).

2.6.4. Indole Test

The indole test determines the ability of an organism to breakdown tryptophan to produce indole. A tiny amount of pure culture was inoculated in the tryptone broth tube. Incubated at 35°C for 24 to 48 hours. To check for indole synthesis, five drops of Kovács reagent were added straight to the tube. A positive indole test results in the creation of a pink to red layer on top of the medium within seconds of applying the reagent. Indole negative cultures result in a yellow or opaque reagent layer (MacWilliams, 2012).

2.6.5. Citrate Utilization Test

The citrate utilization test determines if bacteria can use citrate as their only carbon source for growth. Bacteria were inoculated into tubes with Simmons citrate agar and incubated at 35-37°C. Positive results demonstrate growth and a shift in color from green to blue, indicating citrate utilization. Negative findings indicate no growth or color change (green). Control strains are utilized for quality assurance (MacWilliams, 2009).

2.6.6. Urease production test

The urease production test determines if bacteria have the enzyme urease, which breaks down urea into ammonia and carbon dioxide. Bacteria are added to a urea agar slant and incubated at 35-37°C for 18-24 hours. A positive result indicates a color change to pink or an increase in pH caused by ammonia generation. A negative result indicates no color change or pH increase (Brink, 2010).

2.6.7. Triple sugar iron agar test

The triple sugar iron (TSI) agar test is a differential media used to identify bacteria based on their capacity to ferment sugars and create hydrogen sulfide (H_2S) gas. Using a sterile inoculation needle or loop, streak it down the surface and along the center of the TSI agar slant. Incubate the TSI agar tube at 35-37 °C for 18-24 hours. Color variations in the slant section of the medium were examined, with red indicating no sugar fermentation. The yellow slant denotes the fermentation of one or more sugars (glucose, lactose, or sucrose). The butt section of the medium was monitored for color changes. Red butt suggests that there has been no sugar fermentation. Yellow butt suggests glucose fermentation. Gas generation was indicated by the formation of bubbles or agar rising. The presence of a black precipitate in the agar indicates the generation of hydrogen sulfide (Lehman, 2005).

2.7. Antimicrobial susceptibility testing

The drug susceptibility test for all isolates was performed using the Kirby-Bauer agar disc diffusion method, with inhibition zones measured. It involves spreading bacteria on MHA agar plate, applying antibiotic discs to the surface, and then determining the zones of inhibition following incubation. MHA plates were seeded with 0.5 MacFarland suspension-matched turbidity inoculam. The size of the zones reveals the effectiveness of

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antibiotics. Results are classified as sensitive or resistant. (Khan et al., 2019) The susceptibility profiles were obtained for 15 antibiotic discs including Polymyxins-B(PB,300µg/disk), Piperacillin-Tazobactam (TZP, 100/10µg/disk), Ciprofloxacin (CIP, 5µg/disk), Levofloxacin (LEV, 5µg/disk), Ticarcillin (TIC, 75µg/disk), Imipenem (IPM,10µg/disk), Meropenem (MEM,10µg/disk), Doripenem (DOR, 10µg/disk), Gentamicin (CN, 10µg/disk), Tobramycin (TOB, 10µg/disk), Amikacin (AK, 30µg/disk) Salbactum (SCF, 105µg/disk), Colistin (CN, 10µg/disk), Ceftazidime (CAZ, 30µg/disk), Cefepime (FEP, 30µg/disk).

2.8 Phenotypic screening of virulence factors

2.8.1 Detection of alkaline protease

All bacterial isolates were assessed for their ability to produce protease enzymes (casein hydrolysis) on skim milk agar. The composition of Skim milk agar medium was (per liter) 50g skim milk powder and 23g agar in distilled water. To achieve an alkaline pH, an alkaline buffer, sodium hydroxide, was added to the skim milk agar medium to elevate the pH to an ideal range for alkaline protease activity. Isolates were then streaked on skimmed milk agar and incubated at 37°C for 24 hours. Plates were observed for halo-regions surrounding the streaks. Protease development was confirmed in the clearing zone around the streaks (El-Halim and Nora, 2021).

2.8.2 Detection of lipase

Tween agar was used to test the lipase activity. Tween agar composition includes (per liter) Tween 80 10ml, Peptone 10g, sodium chloride 5g, calcium chloride (CaCl2) 0.1g, agar 15g per liter. Tween 80 agar was inoculated with a single colony of an overnight growth from nutrient agar incubated for (1-5) days at 37°C. The appearance of an opaque and turbid zone surrounding the bacterial growth after 24-48 hours of incubation was interpreted as a positive result, indicating the production of lipase enzymes by the tested bacterial isolates. (Wehedy, 2021).

2.8.3 Detection of phospholipase (Lecithinase)

Egg yolk agar was used for the phospholipase activity. Egg yolk agar was composed of (per liter) 10% (v/v) egg yolk suspension,23g agar. Individual colonies of the bacterial isolates were streaked onto prepared egg yolk agar plates. The plates were then incubated at 35°C for 24 to 48 hours. The formation of a milky white, opaque halo or zone of precipitation surrounding the bacterial growth was interpreted as a positive result for phospholipase (lecithinase) (Wehedy, 2021).

RESULTS AND DISCUSSION

3.1 Isolation and identification of P. aeruginosa

P. aeruginosa is an important cause of community acquired infection as well as nosocomial infection especially in patients admitted in critical care units such as intensive care units and burn care centers. Because of its extensive potential to become resistant to important anti-pseudomonal agents, infection of burn injuries by P. aeruginosa, especially by multi-drug resistant (MDR) strains as well as XDR strains has become a major concern. It is one of the most critical causes of healthcare-related bacterial infection and is responsible for 10% of hospital-acquired infection (Bhatt et al., 2015). Antimicrobial resistance is the main concern among P. aeruginosa strains isolated from wound infections in burn centers(Nikokar et al., 2013). A total of 63 samples were collected from burn patients suspected of having P. aeruginosa infections. the median age of burn patients was 35, ranged from 2 years to 75 years; 27% were children, 40% aged from 18 to 30 and 33% aged more than 50 years There were 61% males and 39% females. The total Body Surface Area (TBSA) affected was 20-50%, with a median TBSA of 30%. The results of bacterial culture obtained from 63 burn's patients admitted by the Burns and Plastic Surgery Center Hayatabad Peshawar, 52 (82.5%) gave positive result while 11 (17.5%) gave negative result.17.5% of cases showed negative culturing that may be due to either patients have antibiotics treatment swabs were obtained from new burns because burn surfaces were initially sterile, but within 48hr the wound was typically colonized by microorganisms. . Similarly, other studies such as Al-Kabi et al (Al-Kabi et al., 2022) and Naqvi et al (Naqvi et al., 2005) , Biswal et al (Biswal et al., 2014) also showed a prevalence of P. aeruginosa infection among burn patients to be 45%, 59.6% and 36.12% while Abbas et al (Abbas et al., 2015) showed a prevalence of 12.54%.

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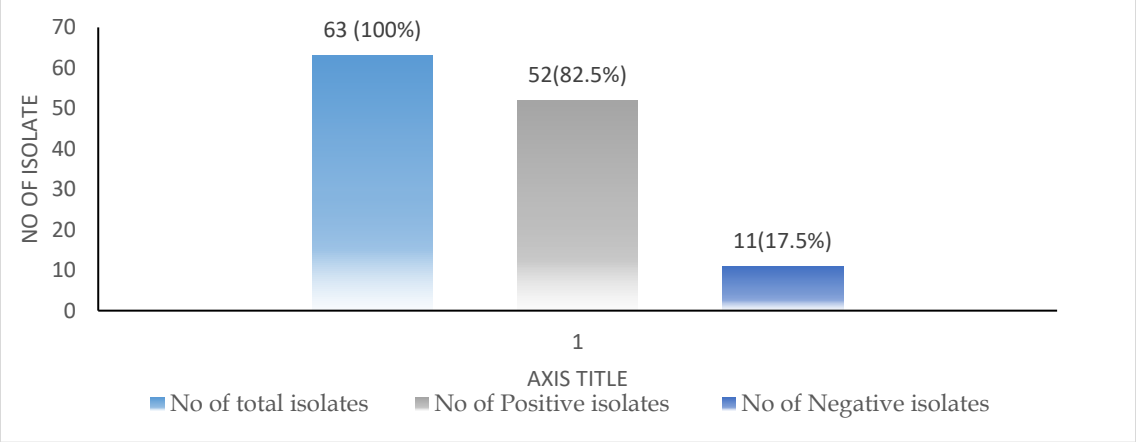

Figure 3.1 Percentage of positive culture and negative culture of burn patients isolates.

3.2 Specie wise distribution of bacteria isolated from burn patients

The species-wise distribution of bacteria isolated from burn patients in this study was analyzed. Among the total positive cultures, 82.5% (52 isolates) consisted of single bacterial isolates. P. aeruginosa was identified as the most prevalent isolate, accounting for 28.8% (15 isolates) of the positive cultures. Following P. aeruginosa, Klebsiella pneumoniae was the second most common isolate, comprising 23.1% (12 isolates) of the positive cultures. Acinetobacter accounted for 17.3% (9 isolates), Staphylococcus aureus for 13.5% (7 isolates), Escherichia coli for 9.6% (5 isolates), and Staphylococcus epidermidis for 7.7% (4 isolates). These findings underscore the clinical significance of these species in burn-related infections. The intrinsic resistance of P. aeruginosa to numerous antibiotics and its capacity to form biofilms present substantial treatment challenges. Klebsiella pneumoniae and Acinetobacter are recognized for their multidrug resistance, while Methicillin-resistant Staphylococcus aureus (MRSA) and Escherichia coli emphasize the importance of appropriate therapy and infection control measures. This information aids in guiding the selection of empirical antibiotics and reinforces the necessity for surveillance, responsible antimicrobial usage, and collaborative efforts in burn care to address the escalating threat of antimicrobial resistance.

Table 3.1. Specie wise distribution of bacteria isolated from burn patients.

Name of bacterial Isolates	Number of bacterial isolates	Percentage (%)
Pseudomonas aeruginosa	15	28.80%
Klebsiella pneumoniae	12	23.10%
Acinetobacter	9	17.30%
Staphylococcus aureus	7	13.50%
Escherichia coli	5	9.60%
Staphylococcus epidermidis	4	7.70%

3.3 Identification of P. aeruginosa by different biochemical tests

Biochemical tests were used to identify P. aeruginosa, which gave a profile characteristic of this species. The following test results were obtained: Gram staining, which was negative, proving that the bacterium is a Gram-negative one. The Indole test was negative. Enzymes catalase and oxidase tested positive, indicating that the bacterium processes enzymes for energy and uses electron transportation. Urease was also positively proved, suggesting that it can hydrolyze urea and use nitrogen. Citrate's test also demonstrated a positive result, suggesting that the bacterium can use citrate as a carbon source. The Triple Sugar Iron also gave a positive result, showing

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red slant and red butt (Red/Red or Alkaline (K)/Alkaline (K)) indicates none of the three sugars are fermented and does not produce gas and does not generate hydrogen sulfide (H2S) which was in same line with findings of a study by Abbas et al (Abbas et al., 2015).These results are consistent with the in vitro features of the bacterium P. aeruginosa and allow us to validate its identification.

Table 3.2 List of biochemical tests used for identification of P. aeruginosa.

S.no	Biochemical Tests	Results
1.	Gram staining	Negative
2.	Catalase	Positive
3.	Oxidase	Positive
4.	Indole	Negative
5.	Citrate	Positive
6.	Urease	Negative
7.	Triple Sugar Iron (TSI)	K/K

Figure 3.2 Catalase tests positive on the right side and Oxidase tests positive on the left side for P. aeruginosa.

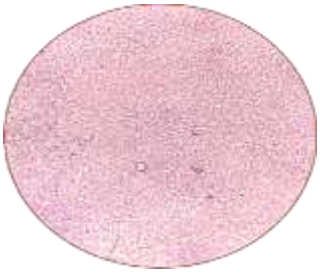

Figure 3.3 Gram staining of P. aeruginosa

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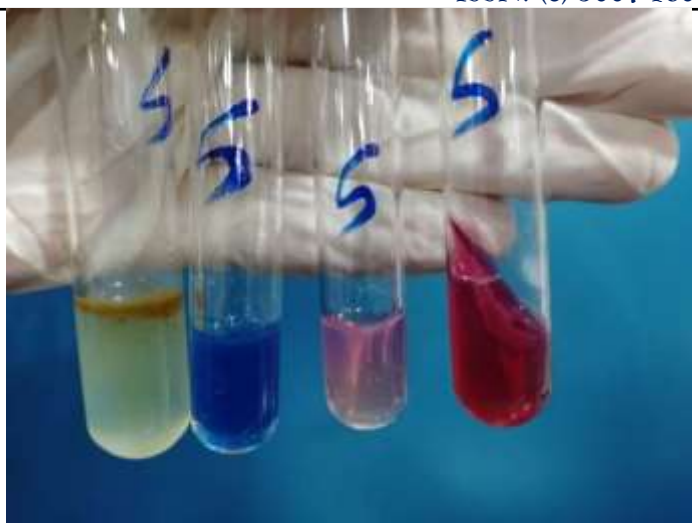

Figure 3.4 Biochemical tests for (From right: TSI K/K, Urease-, Citrate+, Indole-)

3.4 Antibiotic susceptibility pattern of P. aeruginosa

In the present study, the resistance and sensitivity patterns of P. aeruginosa isolates to a variety of antibiotics were thoroughly examined. The study aims to provide a thorough understanding of antibiotic susceptibility patterns, which is critical for developing effective therapy strategies and reducing antimicrobial resistance. The study included 15 different antibiotics, which are Polymyxins-B, Tazobactum, Ciprofloxacin, Levofloxacin, Ticarcillin, Imipenem, Meropenem, Doripenem, Gentamycin, Tobramycin, Amikacin, Salbactum, Colistin, Ceftazidime, Cefepime, each of which was assessed for efficacy against P. aeruginosa isolates. The percentages of resistant and susceptible isolates were cautiously determined for each drug, revealing insight into the prevalence of resistance in the bacterial population. Among the antibiotics tested, Polymyxins are considered the most substantial group of antibiotics against MDR P. aeruginosa. Colistin and Polymyxins-B exhibited high sensitivity showing 93.3% and 80% sensitivity.While other studies such as Gales et al(Gales et al., 2001) reported that Colistin and Polymyxins exhibited 100% sensitivity respectively. Beta-lactamase inhibitors like Tazobactum and Salbactum showed resistance of 40% and 53.3%respectively.while other studies such as Biswal et al (Biswal et al., 2014) showed resistance of 34.5% for Tazobactam. Moderate resistance rates were observed for aminoglycosides, with 53.3% resistance to amikacin, gentamicin and tobramycin. Similarly other studies such as Mohamed and Abdelhamid (Mohamed and Abdelhamid, 2020) reported that P. aeruginosa isolates from burn patients exhibited 62% resistance to gentamycin and 56% resistant to Amikacin and Tobramycin which is slightly higher.Carbapenems, typically the first-line choice for MDR P. aeruginosa infections, displayed concerning resistance rates, with 40% resistance to Imipenem and 46.7% to Meropenem and Doripenem demonstrated highest resistance of 66.7%.However, Bhatt et al.(Bhatt et al., 2015),in their study, found that 61% isolates were resistant to imipenem and 54% were resistant to meropenem.Ciprofloxacin and Levofloxacin both showed 46.7% resistance.While Ceftazidime and cefepime showed 53.3% and 40% resistance,respectively. Other studies such as Wehedy(Wehedy, 2021)exhibited resistance to Ciprofloxacin 68% and Ceftazidime 70% which is considered higher to ours.

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Figure 3.5 Antibiotic susceptibility pattern of P. aeruginosa isolates.

Figure 3.6 Antibiotic susceptibility testing of P. aeruginosa

Out of the 15 samples analyzed, a significant proportion (46.67%) demonstrated extensive drug resistance (XDR), indicating a high level of resistance to multiple classes of antibiotics. Furthermore, 26.67% of the samples were categorized as multidrug-resistant (MDR), indicating resistance to at least three antibiotics within a single class or two from different classes of antibiotics according to previously described criteria, Khosravi et al.(Khosravi et al., 2019). Notably, 26.67% of the samples did not exhibit MDR or XDR, suggesting that they may still be susceptible to various antibiotics. These findings emphasize the significance of antibiotic stewardship and the necessity for effective infection control measures to combat the escalating threat of antibiotic resistance.

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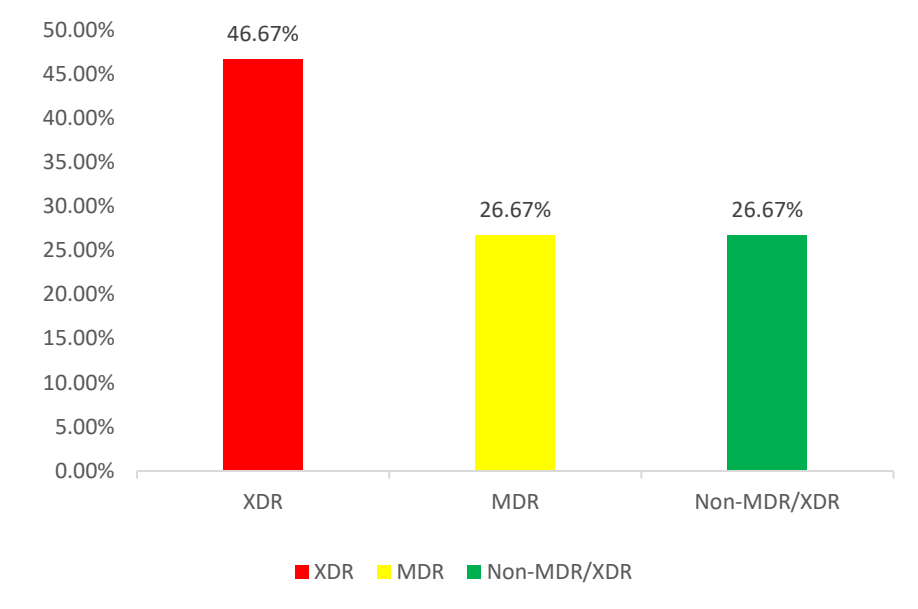

Figure 3.7 Distritbution of drug resistance in P. aeruginosa isolates

3.5 Phenotypic screening of virulence factors of P. aeruginosa

The phenotypic detection results of virulence factors in P. aeruginosa showed a significant incidence of enzymes linked with its pathogenicity. The experiment found that 80% of the P. aeruginosa isolates tested positive for alkaline protease activity. This enzyme plays a necessary role in the degradation of host proteins and evasion of the immune response. The elevated incidence of alkaline protease among the isolates indicates a strong potential for tissue penetration and harm, contributing extensively to the virulence of P. aeruginosa. Lipase activity was detected in 60% of the isolates. Lipases are enzymes that hydrolyze lipids, which are essential components of cell membranes. The existence of lipase advises that P. aeruginosa can effectively degrade host cell membranes, enabling infection and spread within the host. Phospholipase activity was observed in 86.67% of the isolates, the highest among the three virulence factors studied. Phospholipases are enzymes that hydrolyze phospholipids in cell membranes, leading to cell lysis and death. The high percentage of isolates exhibiting phospholipase activity underlines the enzyme's fundamental role in the pathogenicity of P. aeruginosa. Similarly, other studies such as In other studies such as Mohammad and Research(Mohammad and Research, 2013) reported slightly higher percentage in virulence factors expressed by P. aeruginosa isolated from burn patients than our results as phospholipase was 87.50%,100% positivity for lipase and alkaline protease was 68.75%. Hamood et al.(Hamood et al., 1996) suggested that production of high level of phospholipase is important in all types of infections. However, Mun(Mun, 2009) observed the virulence factors of P. aeruginosa isolates from burn patients that alkaline protease was positive for 89.58% followed by lipase which was 87.5% and phospholipase was 79.16% in their sudy.Understanding the frequency and activity of these virulence factors can notify the development of targeted therapeutic policies. For example, inhibitors of these enzymes could be explored as potential actions to mitigate the pathogenic effects of P. aeruginosa.

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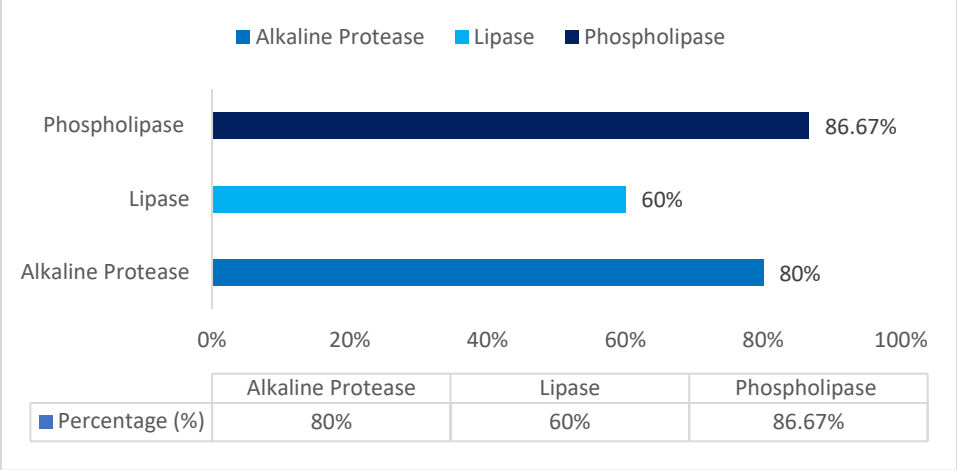

Figure 3.8 Virulence factor of P. aeruginosa isolates

Figure 3.9 Alkaline protease test positive for P. aeruginosa on left side, and negative alkaline protease test on right side.

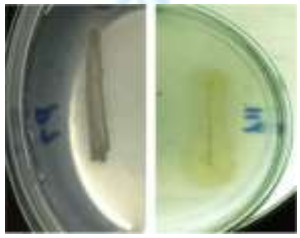

Figure 3.10 Lipase test positive for P. aeruginosa on right side and negative lipase test on left side.

Figure 3.11 Phospholipase test positive for P.aeruginosa on left side and negative phospholipase test on right side.

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3.6 Association of antibiotic resistance with virulence factors

The profiles of enzyme production in the samples revealed a significant association between antibiotic resistance and virulence factors. Among the 4 MDR samples, 75% (3 samples) produced lipase, 50% (2 samples) produced alkaline protease, and all 4 (100%) produced phospholipase, indicating a high possibility of virulence. Similarly, among the 7 XDR samples, 71.43% (5 samples) produced lipase and alkaline protease, and all 7 (100%) produced phospholipase, suggesting a strong association with virulence factors. In contrast, among the 4 non-MDR/XDR samples, 25% (1 sample) produced lipase, 50% (2 samples) produced alkaline protease, and 50% (2 samples) produced phospholipase, indicating a lower likelihood of virulence. These findings imply that antibiotic-resistant isolates are more likely to possess virulence factors, posing a greater risk to public health.

Table 3.3 Resistance association with virulence factors of P. aeruginosa isolates

Resistance Category	Lipase Positive (%)	Alkaline Protease Positive (%)	Phospholipase Positive (%)
MDR Isolates (n=4)	75	50	100
XDR Isolates (n=7)	71.43	71.43	100
Non MDR/XDR (n=4)	25	50	50

CONCLUSION AND RECOMMENDATIONS

In conclusion, this study emphasizes the crucial need for comprehensive measures to control P. aeruginosa infections in burn patients. The high percentage of MDR and XDR strains, associated with their effective virulence factors, requires vigilant surveillance of antibiotics, effective infection control strategies, and constant monitoring. These approaches are critical for mitigating antibiotic resistance and reducing morbidity and mortality in burn care centers. Therefore, further research is highly recommended to identify anti-virulence factors as adjuvant therapies alongside antibiotics for treating P. aeruginosa infections, specifically those demonstrating MDR and XDR profiles. Additionally, studies should be conducted on a larger number of samples and incorporate a wider range of antibiotics classes to better recognize the resistance mechanisms and efficiency of potential treatments.

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