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Molecular Detection of *lasB* Gene in Multidrug-Resistant *Pseudomonas aeruginosa* Isolated from Clinical Specimens

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Abstract: *Pseudomonas aeruginosa* is an opportunistic pathogen, particularly in immunocompromised individuals. Among its many virulence factors, the lasB gene, which encodes for elastase, plays a crucial role in tissue destruction, particularly in acute lung infections and burn wound infections. The study aimed to explore the prevalence of the lasB virulence gene in multidrug-resistant and extensively drug-resistant *P. aeruginosa*. 300 clinical specimens were collected from various hospitals in Babylon City, Iraq. These isolates were obtained from different clinical sources. Specific and differential media cultured all of these specimens. Phenotypic and biochemical tests identified 46 isolates as P. aeruginosa, which was confirmed by the VITEK-2 system. A molecular diagnosis is recognized by the conventional PCR technique to detect the specified gene amplification products of the lasB gene for *P. aeruginosa*. The results showed that *P. aeruginosa* was most prevalent in burn and urine samples, with 46% and 24% rates, respectively. Lower rates were found in wound and sputum samples of 15% and 11%, while the lowest were in ear samples of 4%. Antibiotic resistance was high among the isolates, with 37% being multidrug-resistant (MDR) and 43% extensively drug-resistant (XDR). Only 20% of the isolates were sensitive to antibiotics. The *lasB* gene was predominantly found in drug-resistant strains, with 52% of XDR isolates, 29% of MDR isolates, and 19% of sensitive isolates carrying the gene. The study found a high prevalence of antibiotic resistance among P. aeruginosa isolates, with a significant proportion being multidrug-resistant and extensively drug-resistant. The presence of the lasB gene in nearly half of the isolates a link between the virulence factor and increased resistance mechanisms.

Keywords: *Pseudomonas aeruginosa*, *lasB* gene, MDR, XDR

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Introduction

Pseudomonas aeruginosa is an important clinical agent, as this bacterium is an opportunistic pathogen that can cause a wide range of acute and chronic injuries and diseases in humans (1). These bacteria can resist disinfectants and thus play a role in hospital-acquired infections (2). Exacerbating the challenge with *P. aeruginosa* infections is this organism high intrinsic and acquired resistance to many current antibiotics (3). Because of this challenge and the severity of infections caused by *P. aeruginosa*, it has been placed among the priority pathogens, known as the ESKAPE, for which new antimicrobial development is urgently needed according to the World Health Organization (WHO) and the Centers for Disease Control (CDC) (4, 5).

Pseudomonas aeruginosa infections are particularly challenging due to their

high level of resistance to antibiotics (6). Antibiotic resistance in *P. aeruginosa* can be divided into intrinsic and acquired resistance. And adaptive resistance. Given the presence of intrinsic and acquired resistance modes in P. aeruginosa, it is challenging to cure any resulting infections (7). Multidrug-resistant bacteria were considered non-susceptible to at least one antimicrobial agent in three or more antimicrobial classes (8). Multidrug resistance can arise in two main ways. The first is the concentration reduction of antimicrobial compounds in the cell, either by lowering the membrane permeability or actively exporting these compounds by specialized transporters. The second is by sequentially accumulating many genes or mutations that cause resistance to individual antibiotics, which is a very slow process (9). The high prevalence of *P. aeruginosa* in hospitals, combined with the overuse of broad-spectrum antibiotics, has led to a significant surge in drug-resistant *P. aeruginosa*. As a global problem, the increasing rate of multidrug-resistant *P. aeruginosa* (MDR-PA) strains has complicated medical therapy against P. aeruginosa (10). The global incidence of infections due to MDR-PA has been increasing; this includes *P. aeruginosa* strains resistant to carbapenem (CR-PA), which are particularly difficult to treat (11).

The virulence factors appear on the surface of *P. aeruginosa* or are excreted by it (12). Large quantities of Elastase are produced. It includes *LasB* zinc metalloprotease (elastase), *LasA*metalloendopeptidase, and alkaline protease(13).*LasB P. aeruginosa* releases a wide range of extracellular proteases critical for invasion in acute infections: *LasA* and *LasB* elastases (14). The *T2SS secretes LasB elastases* under the regulation of QS systems (15) and degrades host elastin (16). It is the most abundant protease and the principal extracellular virulence factor (14).It also degrades exogenous flagellin under calcium-replete conditions, avoiding TLR5 recognition (17). Apart from its elastinolytic activity, it also disrupts epithelial tightjunctions (18)and cleaves other host proteins, for instance, surfactant proteins (SP-A and SP-D), cytokines (TNF-_, IFN-, IL-6 or IL-2), immunoglobulins and components of the inflammasome(14), thereby interfering with bacterial clearance.This study aimed to investigate the prevalence of the *lasB* virulence gene in MDR and XDR*Pseudomonas aeruginosa* isolates collected from clinical specimens in Babylon City, Iraq. Furthermore, it aimed to assess the antibiotic resistance profiles of these isolates and to investigate a possible association between the presence of the lasB gene and the mechanisms of antimicrobial resistance.

Materials and Methods

Collection of samples

This study included 300 clinical specimens collected from patients of different ages and genders, from various clinical sources, including 99 from burn swabs, 73 from urine samples, 4 from blood, 41 from sputum, 55 from wounds, and 28 from the ear. The specimens were collected using swabs, transported in containers with sterilized transport medium, and transferred to a research laboratory. The clinical specimens were collected from 7 January 2025 to 23 March 2025, from various hospitals in Babylon City, including Al-Hilla Teaching Hospital, Imam Sadiq Teaching Hospital, Imam Ali Hospital, Babylon Hospital for Women and Children, and Murjan

Medical City. The Ministry of Health has granted ethical approval.

Identification of *P. aeruginosa* Isolates

The clinical specimens were immediately inoculated in MacConkey agar, blood agar, and nutrient agar and incubated aerobically overnight at 37°C, followed by subculture on Cetrimide agar, followed by microscopic examination and biochemical identification, and utilized the VITEK 2 system to ensure thorough identification.

Antibiotic susceptibility test

Antibiotic susceptibility testing is done once the microorganism is identified. The susceptibility test of P. aeruginosa isolates to antibiotics such as Piperacillin, Cefazolin, Ceftazidime, Cefepime, Imipenem, Meropenem, Amikacin, Gentamicin, Ciprofloxacin, and Levofloxacin was examined. Susceptibility to antibiotics was determined by the Vitek 2 system, which automatically measures a turbidity signal for each test well containing an antibiotic, every 15 minutes for up to 18 hours. These data are used to generate growth curves and by comparing with a control, the minimum inhibitory concentration (MIC) of each antibiotic is estimated (22). MIC results (µg/ml) were translated into clinical categories (Susceptible, Intermediate, and resistant) by comparing with breakpoints for susceptibility category determination recommended by the Clinical and Laboratory Standards Instituteguidelines.

Molecular study

Extraction of Genomic DNA

DNA is extracted from examined isolates using a commercial purification system (Presto™ Mini gDNA Bacteria Kit, Geneaid Biotech Ltd, Taiwan) following the manufacturer instructions; this kit was designed to isolate DNA from Gram-positive and Gram-negative bacteria. Furthermore, samples were analyzed on a 1% agarose gel to screen for genomic DNA before storing at -20°C for future molecular investigations.

Molecular diagnosis of *LasB*gene

The virulence gene elastase (lasB) in P. aeruginosa isolates was identified through PCR. The existing study used a couple of primers for the gene. The primer sequences for lasBwere F-5-GGAATGAACGAAGCGTTCTC-3 and R-5-GGTCCAGTAGTAGCGGTTGG-3 with amplification band sizes of 300bp (24), which were supplied by Macrogen Company, Korea, in a lyophilized form.

PCR Components

TransGen Biotech, China, provided the PCR kit used in this study. The PCR reaction mixture consisted of 12.5 µl master mix, 1µl Forward Primer (10 picomole), 1µl Reverse Primer (10 picomole), 8.5µl De-ionized water, and 2µl the DNA (Total volume 25 µl). A QuantusFluorometer was used to detect the concentration of extracted DNA anddetermine the samples' goodness for downstream applications.

PCR Program

Optimization of PCR reaction is accomplished after several trials, thus, the following program for amplifying a fragment of Las B (300) was adopted, initial denaturation was done at 95°C for 3 min, followed by 30 cycles of reaction having the steps of denaturation at 95°C for 30 secs, annealing at 58°C for 30 secs, extension at 72°C for 1 min, thenafter, the final extension step was set at 72°C for a 3 min.Detection of the amplified genes was done by horizontal agarose gel electrophoresis. The agarose gel (1.5%) was stained with ethidium bromide and subsequently exposed to UV light. The

optimal product size was confirmed by comparing it with the 100-1500 bp DNA ladder.

Results and Discussion

Results

Morphological and cultural characterization tests showed that 46 *P. aeruginosa* isolates were identified from all the different clinical specimens (300) collected. The results indicate that *P. aeruginosa* infections are most prevalent in burn patients, accounting for 46% of cases, followed by urine (24%) and wound (15%) samples. Sputum and ear samples show lower percentages (11% and 4%, respectively), while no cases were detected in blood samples. Further investigation into infection sources and prevention strategies is warranted. Pronounced statistics can be observed in Table 1.

Table 1 summarizes the clinical sources of *P. aeruginosa* isolates, highlighting their prevalence across different specimen types. Among 300 clinical specimens, 46 isolates were confirmed as *P. aeruginosa*. Burn swabs constituted the predominant source (46%, n=21), followed by urine (24%, n=11), wound swabs (15%, n=7), sputum (11%, n=5), and ear samples (4%, n=2). No isolates were detected in blood specimens. The chi-square analysis revealed a statistically significant association between specimen type and P. aeruginosa prevalence ($P \leq 0.05$). These findings align with prior studies indicating that burns and wounds are high-risk sites for *P. aeruginosa* colonization due to compromised host defenses and prolonged hospitalization. The absence of isolates in blood samples suggests bacteremia caused by this pathogen is rare in the studied population, contrasting with other regions where bloodstream infections are more frequently reported.

The identification of P. aeruginosa isolates is usually based on cultural properties, the presence of characteristic pigments, and growth at 42°C. All the isolates were cultured on MacConkey agar, blood agar, nutrient agar, and Cetrimide agar. All 46 isolates were Gram-negative, rod-shaped, and red-colored under the microscope. All isolates showed the ability to grow at 42 °C.

Table(1): Distribution of *Pseudomonas aeruginosa isolates based on the* source of isolation.

Clinical Sample	Totalspecimens	*Number of p. aeruginosa*	Percentage from *p. aeruginosa*
Burn	99	21	46%
Urine	73	11	24%
Sputum	41	5	11%
Blood	4	0	0%
Wound Swabs	55	7	15%
Ear	28	2	4%
Total	300	46	100%
P-value	---	---	0.039 *

Chi-square;* ($P \leq 0.05$).

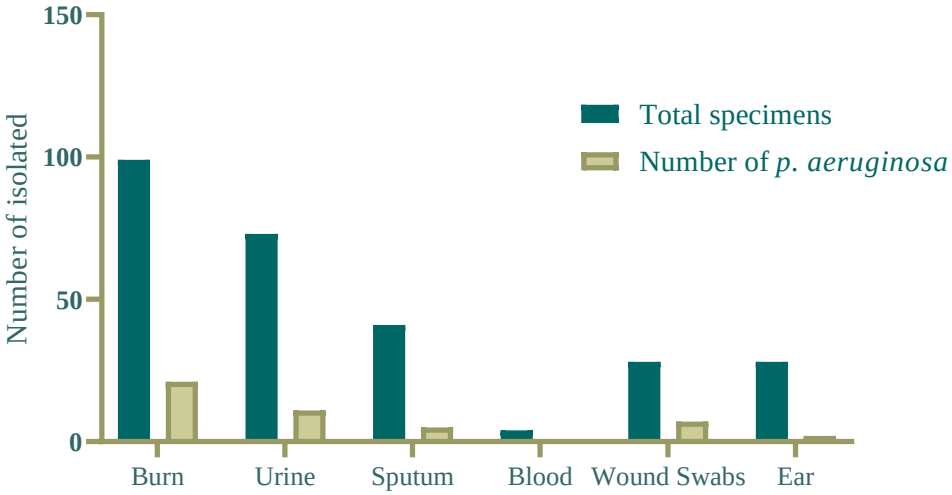

Figure (1):Distribution of Pseudomonas aeruginosa isolates among different clinical specimens.

This investigation showed that *P. aeruginosa* was highly isolated from burn specimens, which is the place where*P. aeruginosa* can reach the easiest (21, 46%). With a high prevalence of *P. aeruginosa* in the community, this outcome was anticipated. It may be linked to the growing number of immunocompromised patients in our population due to various illnesses and environmental contaminations in hospitals and the country, particularly among patients who require prolonged hospital stays. This is consistent with research by (23), which discovered that 20 isolates (25%) were recognized as *P. aeruginosa* based on morphological and culture characterization tests. (24) discovered that *P. aeruginosa* isolates were present in 71 out of 110 (64.55%) burn patients. The isolation rate of *P. aeruginosa* from wounds was 7 (15%), comparable to the findings of (25), who found that the prevalence of *P. aeruginosa* in wound samples was 14.5%. The current study's 5 (13.75%) *P. aeruginosa* isolation rate from sputum is similar to (26) study, which found that the prevalence of *P. aeruginosa* in sputum samples was 13.3%, additionally, lung infections like pneumonia are another important clinical manifestation of this pathogen, particularly in patients with cystic fibrosis or chronic obstructive pulmonary disease (COPD), as evidenced by the lowest percentages 2 (4%), which were found in the (27) study. Urine samples comprised 11 (23.91%) isolates in the current study. *He suggests that P. aeruginosa-caused urinary tract infections (UTIs) are also prevalent*. This is especially important given the prevalence of catheter-associated UTIs in hospital settings. Blood samples used in this investigation reveal no isolates. However, another study (27) only found 1 (12.5%), suggesting that *P. aeruginosa*-caused bacteremia (bloodstream infections) are uncommon in this study. The lowest percentage (4%), compared to 13 (26%) in an Iraqi study (27), indicates that ear infections brought on by this virus are less frequent. The abuse of antibiotics may explain this discrepancy, as well as different hospital infection control strategies, sanitary conditions, and local environment. It may also result from the different sample sizes for these bacterial isolates in the current investigation.

Generally,various factors, such as changes in target site architecture and cell membrane permeability, are responsible for the resistance of various antibiotics (28).

To confirm the diagnosis, the Vitek-2 system also identified a total sample of presumptive P. aeruginosa positive for cultural methods tests.The mentioned tests and Vitek-2 system tests (Table 1) confirmed that 46 isolates out of 300 clinical specimens belonged to P. aeruginosa.

Forty-six isolates of *P. aeruginosa* were tested for their antibiotic susceptibility toward 10 antibiotics using the Vitek-2 system (Table 2). The current study revealed a remarkable increase in *P. aeruginosa* resistance to the antibiotics used in this study, but especially to beta-lactam antibiotics, represented by penicillins such as piperacillin. Resistance to this antibiotic has reached 83%, with no intermediate cases, and only 17% sensitivity. Cefazolin follows with 74% resistance and 26% sensitivity. Ceftazidime and Cefepime exhibit similar resistance levels of 65% and 61%, respectively, with minor intermediate cases (7% and 6%) and moderate sensitivity (26% and 33%). Carbapenems, Imipenem and Meropenem, show lower resistance rates (35% and 37%) and higher sensitivity (59% and 61%), making them more effective options. Amikacin and Gentamicin, both aminoglycosides, display resistance of 59% and 67%, and sensitivity of 28% and 26%, respectively, with Amikacin having a higher intermediate rate (13%). Fluoroquinolones, Ciprofloxacin and Levofloxacin, show similar resistance patterns (61% and 67%), with low intermediate rates (4% and 2%) and moderate sensitivity (35% and 31%). The highlights significant resistance to commonly used antibiotics like Piperacillin and Cefazolin, while carbapenems (Imipenem and Meropenem) remain relatively effective. Notably, 37% of isolates were classified as extensively drug-resistant (XDR), and 36.96% as multidrug-resistant (MDR). Statistical significance ($P \leq 0.01$) across all antibiotics emphasizes the alarming resistance trends. These results corroborate global reports of rising MDR/XDR P. aeruginosa strains, driven by efflux pump activity, membrane impermeability, and β-lactamase production.

Table(2): Antimicrobial Susceptibility of Pseudomonas aeruginosa Isolates to Different Antimicrobial Agents.

Antibiotic	Resistant	Intermediate	Sensitive
Piperacillin	38 (83%)	0 (0.0%)	8 (17%)
Cefazolin	34 (74%)	0 (0.0%)	12 (26%)
Ceftazidime	30 (65%)	3 (7%)	13 (28%)
Cefepime	28 (61%)	3 (6%)	15 (33%)
Imipenem	16 (35%)	1 (3%)	27 (59%)
Meropenem	17 (37%)	1 (2%)	28 (61%)
Amikacin	27 (59%)	6 (13%)	13 (28%)
Gentamicin	31 (67%)	3 (7%)	12 (26%)
Ciprofloxacin	28 (61%)	2 (4%)	16 (35%)
Levofloxacin	31(67%)	1 (2%)	14 (31%)
P-value	**<0.0001 ****		

Chi-square; ** (P≤0.01).

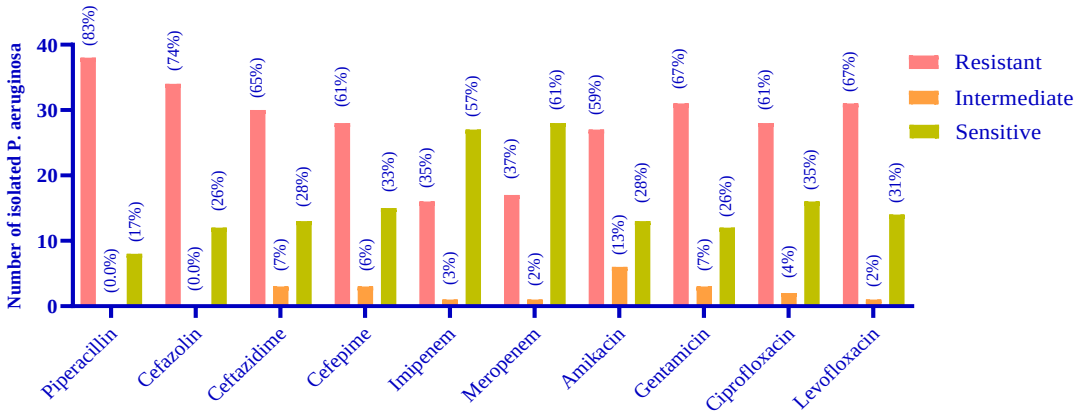

Figure (2):Antibiotic susceptibility pattern of Pseudomonas aeruginosa isolates.

These findings concurred with research published by (23). According to the current findings, P. aeruginosa shows high levels of resistance to five antibiotics: levofloxacin, cefazolin, ceftazidime, gentamicin, and piperacillin. According to (29),the processes restricting intracellular drug concentrations include reduced outer membrane permeability, decreased cytoplasmic membrane absorption, and active efflux back across the cytoplasmic membrane. *P. aeruginosa* intrinsic antibiotic resistance may be caused by the production of chromosomally encoded efflux pumps, which explains these high resistance results (Li and Nikaido, 2004). Antibiotic resistance is generally ascribed to various factors, such as changes in target site architecture and increased cell membrane permeability (28).

The PCR amplification showed that the lasB genes were found in 21 (45.65%) isolates, with amplicons of 300 bp on the agarose gel (Figure 1).Table 3 indicates the presence of the *lasB* gene in 4 (19%) susceptible isolates, 6(29%) MDR isolates, and 11(52%) XDR isolates out of 46 isolates

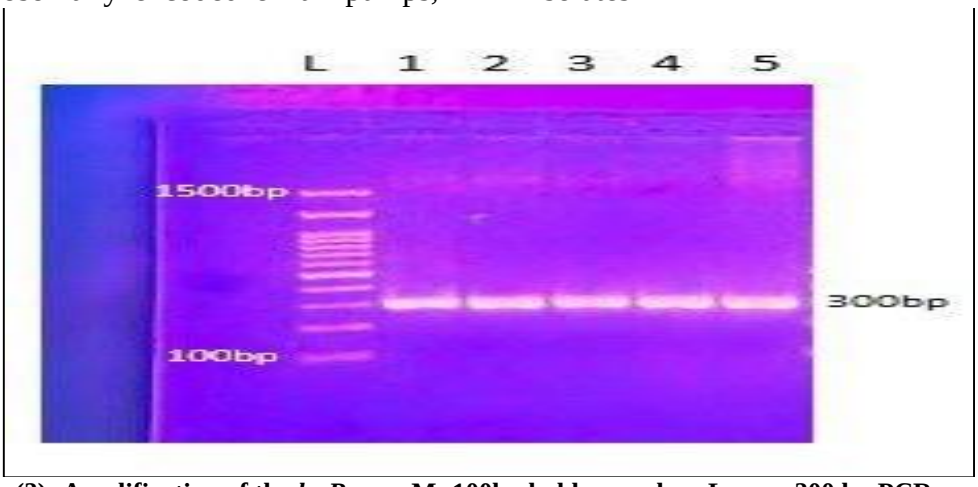

Figure (3): Amplification of the *lasB*gene. M: 100bp ladder marker. Lanese 300 bp PCR products

Table 3 correlates the presence of the *lasB*virulence gene with resistance phenotypes. The lasB gene was detected in 45.65% (21/46) of isolates, with a higher prevalence in XDR (55%, n=11) compared to MDR (35.29%, n=6) and susceptible isolates (44.44%, n=4). Chi-square analysis confirmed a significant

association between *lasB* presence and resistance category ($P \leq 0.05$). The dominance of lasB in XDR strains (52.38% of total lasB-positive isolates) suggests a potential interplay between virulence and resistance mechanisms. This aligns with hypotheses that hypervirulent strains may co-select for resistance under antibiotic pressure, enhancing survival in hostile environments. The findings support the need for dual strategies targeting both virulence (e.g., elastase inhibitors) and resistance mechanisms (e.g., efflux pump blockers) to combat recalcitrant P. aeruginosa infections.

Table (3): Frequency of *lasB* gene of *P. aeruginosa* according to the type of resistance.

Type of resistance	S	MDR	XDR	Total	P-value
No. of*P. aeruginosa*isolate	9	17	20	46	0.03*
***lasB* gene presence**	4	6	11	21	

Chi-square;* (P≤0.05).

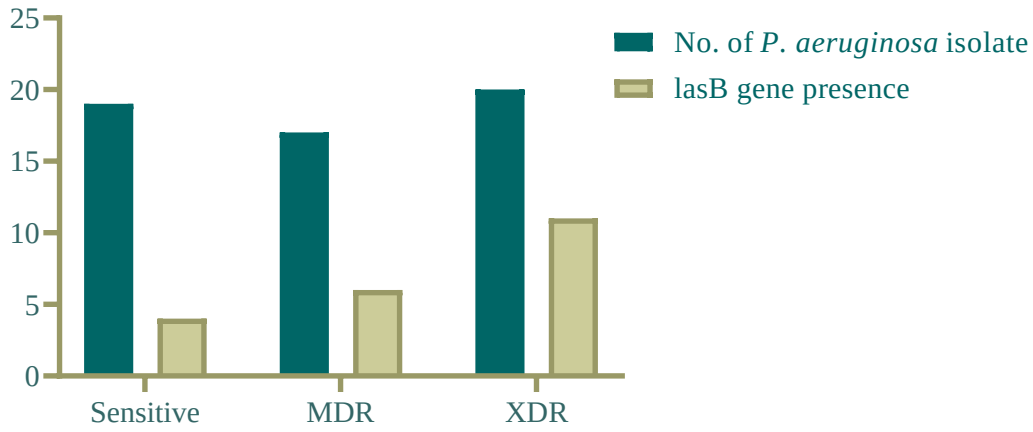

Figure (4): Distribution of lasB gene among Pseudomonas aeruginosa isolates with different resistance profiles.

Based on the results of the current investigation, the *lasB* gene, which has an amplified size of 300 bp, was found in 21 (45.6%) of the 46 isolates of *P. aeruginosa* (Table 3). This outcome is similar to earlier investigations that used the same primers for the *lasB* gene and produced a band with an identical molecular weight of 50% (24, 30). Using the same primers for the *lasB* gene, (31) also produced a band with an identical molecular weight of 82%. Several factors affect the frequency of *P. aeruginosa* and the percentage of virulence factor genes, including environmental factors, patient immunological health, contamination levels, strain type, and virulence (32). Numerous extracellular components that promote survival and heightened virulence are also secreted by *P. aeruginosa*(33). 35 of the 46 *P.aeruginosa* strains possessed more than two virulence factors, and the XDR group was more active than the MDR strains, according to (34). Another known factor contributing to *P. aeruginosa* multi-drug resistance is biofilm (35).While *lasA* genes were absent from all strains, *lasB* genes were present in all strains (36).

In addition, several*LasB*gene variations with varying molecular weights and substrate specificities were found, indicating that *P. aeruginosa* possesses a wide variety of *LasB*

enzymes that have developed to accommodate various hosts and conditions (37). The distribution of *lasB* across resistance types reveals notable trends. Among susceptible (S) isolates, *lasB* was found in 4 out of 9 isolates(44.44%), while in MDR isolates, it was detected in 6 out of 17 (35.29%). In contrast, XDR isolates showed the highest prevalence of *lasB*, with 11 out of 20 isolates (55%) testing positive. When considering the percentage contribution to the total *lasB*-positive isolates, S, MDR, and XDR accounted for 19.04%, 28.57%, and 52.38%, respectively. This suggests that XDR isolates are more frequently linked to *lasB* than S and MDR isolates. The results suggest a possible connection between virulence factors and antibiotic resistance. The increased *lasB* prevalence in XDR isolates might suggest that these strains have developed defense mechanisms to increase their pathogenicity, perhaps to survive in harsh conditions like high antibiotic pressure. This supports the idea that more resistant bacteria might also have extra virulence factors to help them survive and proliferate.

All 46 clinical isolates of *P. aeruginosa* tested in this study were classified into groups based on their antibiotic resistance. Table 3 showed several isolates of Multi-Drug Resistance (MDR). MDR is defined as the resistance of a bacterial isolate towards antimicrobial drugs from at least three antipseudomonal classes of the drugs used in this study. In contrast, extensive drug resistance (XDR) means an isolate's resistance to all but one or two courses of antibiotics (38). Of the 46 *P. aeruginosa* isolates, 17were MDR, 20 were XDR, and 9 were sensitive. 23 were shown to be MDR, substantially linked with older (>65) patients by (34). According to (39) research, *P. aeruginosa* strains from European nations had a lower rate of MDR occurrence. Public health is seriously threatened by the development and spread of antibiotic resistance in bacterial pathogens (40). The overuse of antibiotics may cause*P. aeruginosa*to develop resistance to many of them. This would result in the development of multidrug-resistant (MDR) *P. aeruginosa*and the buildup of antibiotic resistance and cross-resistance (41). Based on the definition of MDR, 17(36.96%) isolates were confirmed as MDR,which agreed with (27).

Conclusion

The study revealed a high prevalence of antibiotic resistance among P. aeruginosa isolates, with a substantial proportion classified as multidrug-resistant and a considerable fraction exhibiting extensively drug-resistant phenotypes. The lasB gene was detected in nearly half of the isolates, with a majority observed in extensively drug-resistant strains, suggesting a potential association between the presence of this virulence factor and increased resistance mechanisms. These findings emphasize the critical challenge posed by P. aeruginosa infections, which concurrently demonstrate heightened virulence and resistance profiles, and necessitate comprehensive approaches integrating antimicrobial stewardship, virulence-targeted therapies, and infection control measures to address such dual-threat pathogens effectively.

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