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A study on antibiotic resistance and pigment production by locally isolated *Pseudomonas aeruginosa*

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ABSTRACT

This study aimed to investigate the phenotypic and antibiotic resistance characteristics of Pseudomonas aeruginosa isolates obtained from various clinical specimens. A total of 55 isolates were recovered from 390 samples, with the highest recovery from ear swabs, burns, and wound infections. The identification of *P. aeruginosa* was confirmed using morphological, biochemical, and selective culture methods. All isolates were Gram-negative, motile rods, oxidase- and catalase-positive, and demonstrated characteristic growth on cetrimide and MacConkey agar. IMViC testing showed negative results for indole, methyl red, and Voges–Proskauer, while citrate was utilized by all isolates. Antibiotic susceptibility profiling revealed variable resistance patterns. The highest resistance was noted for ticarcillin-clavulanate (98.18%) and cefepime (78.18%), while piperacillin-tazobactam showed the lowest resistance (18.18%). Alarmingly, colistin resistance was detected in 96.36% of the isolates based on MIC values (≥ 4 µg/mL), raising serious clinical concerns. Furthermore, 87% of the isolates exhibited multidrug resistance (MDR), and 18% were classified as extensively drug-resistant (XDR), reflecting a significant threat to treatment efficacy. All isolates produced β-hemolysin on human blood agar and 100% of selected strains synthesized pyocyanin on King A agar, confirming the presence of key virulence factors. The high rates of resistance and virulence indicate an urgent need for surveillance, antibiotic stewardship, and the development of alternative treatment strategies. These findings contribute to the growing body of evidence on the clinical challenges posed by *P. aeruginosa* in healthcare settings.

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1. INTRODUCTION

Pseudomonas aeruginosa is among the most adaptable bacterial species known to thrive in harsh environments. It stands as a prominent example of an opportunistic pathogen that exploits weakened immune defenses in humans to initiate infections that may lead to severe clinical outcomes [1]. This organism is distinguished by its low intrinsic susceptibility to a wide range of antibiotics and is recognized as the second most common opportunistic pathogen and a major cause of healthcare-associated infections. It frequently affects individuals with compromised immunity, such as patients with cystic fibrosis, burn injuries, diabetic foot ulcers, chronic wounds, or otitis externa [2]. The phenotypic and genetic traits of *P. aeruginosa* are subject to continuous modification, particularly in strains isolated from cystic fibrosis patients, where selective environmental pressures drive adaptive evolution. These changes enhance its pathogenic potential and antibiotic resistance, offering a high degree of flexibility for survival under hostile conditions in clinical settings, especially in intensive care units.

aeruginosa is a leading cause of nosocomial infections and is associated with significant morbidity and mortality, particularly in patients with burn wound infections and ventilator-associated pneumonia [3].

The bacterium's pathogenicity arises from multiple virulence mechanisms. These include biofilm formation and the secretion of several exotoxins, including Exotoxin A, which inhibits host protein synthesis; Exotoxin S, which contributes to immune cell dysfunction; and Exotoxin V, which contributes to cellular destruction. In addition, *P. aeruginosa* produces a variety of lytic enzymes such as proteases, ureases, and hemolysins, as well as pigments like pyocyanin that further enhance its cytotoxic effects and ability to persist in the host environment [4]. Gnomically, *P. aeruginosa* possesses more than six million base pairs, which contributes to its exceptional capacity for environmental adaptation, antimicrobial resistance, and persistence in immunocompromised hosts [5]. This large genome supports frequent genetic rearrangements and regulatory flexibility, enabling the bacterium to adjust to different ecological niches and promote infection [6]. Such adaptations increase its resistance to antimicrobial agents and enhance detoxification mechanisms, making it one of the most difficult pathogens to treat [7]. One of its major immune evasion strategies involves secreting virulence-related proteins directly into the host cytoplasm or extracellular space, thereby impairing immune surveillance [8].

Multidrug resistance (MDR) is another alarming feature of *P. aeruginosa*, defined as resistance to at least three different antibiotic classes—most commonly β-lactams, aminoglycosides, and fluoroquinolones. This resistance is often mediated by mobile genetic elements such as integrons and plasmids. Integrons may carry genes encoding resistance to a wide spectrum of antimicrobials, including β-lactams—the largest and most widely used class of antibiotics—alongside aminoglycosides and other agents [9]. The growing threat of infections caused by MDR *P. aeruginosa* is influenced by several temporal and spatial factors, including environmental conditions, the number of colonized patients in the same hospital unit, the ratio of pathogen carriers to susceptible patients, compliance with infection control measures, and antibiotic usage practices [10].

2. Experimental Methodology:

2.1. Specimens' collection:

Between September 10, 2024, and January 20, 2025, a total of 390 clinical specimens were collected from patients presenting with various infectious and inflammatory conditions. These included urine samples, swabs from otitis media, sputum, burn and wound swabs, and blood samples. All specimens were obtained from patients attending Baquba Teaching Hospital and the outpatient consulting clinic in the Diyala region. The collected specimens were processed using conventional microbiological techniques in accordance with established diagnostic protocols.

2.2, Microscopic and Biochemical Identification

Initial identification of bacterial isolates was performed using standard microscopic and biochemical methods. Blood agar, MacConkey agar, King A agar, and Cetrimide Agar were used to study the phenotypes of *P. aeruginosa* colonies, such as colony shape, color, and size, as well as the kind of hemolysis. Gram staining was conducted to assess cell morphology, Gram reaction, and cellular arrangement. Biochemical characterization included IMViC, oxidase, and catalase tests, following conventional microbiological protocols [11].

2.3. Screening for Pigment Production

The ability of *P. aeruginosa* to produce characteristic pigments was evaluated using King A and King B media. King A agar was employed to detect the production of pyocyanin, the blue-green pigment typically associated with this species, while King B agar was used to promote the synthesis of pyoverdine and concurrently suppress pyocyanin expression, thereby aiding in the differential observation of pigment profiles.

2.4. Antibiotic Susceptibility Testing

The antimicrobial susceptibility of the isolates was determined using the Kirby–Bauer disk diffusion method, following Clinical and Laboratory Standards Institute (CLSI) guidelines [12]. Standardized bacterial suspensions were inoculated on Mueller–Hinton agar, antibiotic disks were applied, and plates were incubated at 37°C for 24 hours. Inhibition zones were measured and interpreted according to CLSI breakpoints.

2.5. Determination of Minimum Inhibitory Concentration (MIC) and Sub-MIC Levels

To evaluate the efficacy of colistin against *P. aeruginosa*, MIC and sub-MIC values were determined through broth microdilution assays. Serial dilutions of colistin were prepared in Mueller–Hinton broth, and bacterial growth was assessed after incubation at 37°C. The MIC was defined as the lowest concentration that inhibited visible growth, while sub-MIC values represented concentrations below the MIC threshold that still permitted limited bacterial proliferation [13].

3. RESULTS AND DISCUSSION

3.1. Identification of *P. aeruginosa* Isolates

Microscopic examination of Gram-stained bacterial smears revealed the presence of Gram-negative rod-shaped cells, consistent with the morphological features of *P. aeruginosa*. These findings align with previously reported observations [11]. On MacConkey agar, the bacterial colonies appeared large, convex, and mucoid with irregular margins and a characteristic odor. The colonies were pale in color because the organism was unable to ferment lactose. On blood agar, β-hemolysis was observed, indicating the bacterium's ability to produce hemolysin, which contributes to its virulence through red blood cell lysis [11]. In addition, growth on cetrimide agar, which contains 0.03% cetrimide, confirmed the identity of the organism; the medium selectively supports the growth of *P. aeruginosa* while inhibiting other species. The isolates produced a greenish-blue pigment, identified as pyoverdine, in line with findings from similar studies in Egypt [14].

Motility was evaluated using semi-solid agar by stab inoculation. Diffuse growth surrounding the inoculation line indicated the presence of a polar flagellum, a known feature of *P. aeruginosa* that facilitates active motility [15].

3.2. Biochemical Characterization

Biochemical testing further supported the identification of the isolates. All isolates demonstrated strong oxidase and catalase activity. The oxidase test showed a rapid color change to dark purple, reflecting the organism's ability to transfer electrons to a chromogenic substrate through cytochrome oxidase [11]. In the catalase test, vigorous bubbling was observed upon the addition of hydrogen peroxide, confirming catalase production that degrades hydrogen peroxide into water and oxygen [11]. Regarding the IMViC profile, all isolates were negative for indole, methyl red, and Voges–Proskauer tests, while citrate utilization was positive.

3.3. Distribution of Isolates by Clinical Source

Among the 390 clinical samples examined, 55 *P. aeruginosa* isolates were recovered, representing 14% of the total specimens. The highest isolation rate was observed in ear swabs (5%), followed by burns (2.5%), wounds (2%), sputum (1.7%), urine (1.5%), and blood (1%) (Table 1). These results highlight the organism's prevalence in otic and burn-related infections.

Table (1): Distribution of isolates by clinical source

No	Sample Source	samples	*P. aeruginosa* Isolates	Percentage ratio
1	Ear	47	20	5%
2	Burns	22	10	2.5%
3	Wounds	60	8	2%
4	Sputum	50	7	1.7%
5	Urine	100	6	1.5%
6	Blood	111	4	1%
	Total	390	55	%14

3.4. Antibiotic Susceptibility Profiles

Antimicrobial susceptibility testing was performed on all 55 isolates using the Kirby–Bauer disk diffusion method. The isolates exhibited variable resistance patterns across the tested antibiotics (Table 2). Notably, the highest resistance was recorded against Ticarcillin-clavulanate (98.18%), followed by Cefepime (78.18%) and Aztreonam (34.55%). Resistance to carbapenems was moderate, with 30.91% and 21.82% resistance rates against Imipenem and Meropenem, respectively. The lowest resistance was observed against Piperacillin-Tazobactam (18.18%).

Table (2): Antibiotic susceptibility profile of *P. aeruginosa*

N	Antibiotic	Percentage ratio	N	Antibiotic	Percentage ratio
1	Ticarcillin- clavulanate	98.18%	6	Piperacillin	27.27%
2	cefepime	78.18%	7	Norfloxacin	23.64%
3	Aztreonam	34.55%	8	Meropenem	21.82%
4	Levofloxacin	32.73%	9	Tobramycin	21.82%
5	Imipenem	30.91%	10	piperacillin-Tazobactam	18.18%

The antimicrobial susceptibility testing of *P. aeruginosa* isolates revealed noticeable variation in resistance levels across the antibiotics tested. Resistance to piperacillin, a broad-spectrum penicillin, was detected in 27.27% of isolates. This finding is comparable to previous reports by Zainab (2017), which documented resistance rates of 20%, yet it contrasts with the study by Najeeb (2020), where no resistance to piperacillin was observed [16–17]. High resistance was also observed against cefepime, a fourth-generation cephalosporin, with 78.18% of isolates showing resistance. This aligns closely with the findings of Alsaadi (2020), who reported a resistance rate of 80.24% [18]. However, this figure is markedly higher than those reported by Pérez et al. (2020), who recorded lower resistance rates of 43.4%, respectively [19]. Notably, the highest resistance rate in this study was observed for ticarcillin-clavulanate, reaching 98.18%. This result is consistent with the study by Mohamed and Al-Taai (2023) in Diyala Province, where 100% resistance was recorded [20]. Comparable results were also reported by Al-Sajad and Alsalim (2024), with a resistance rate of 92.86% [21]. The high resistance is likely due to overproduction or structural modification of β-lactamases in P. aeruginosa, thereby reducing the effectiveness of both ticarcillin and the β-lactamase inhibitor clavulanate.

Resistance to piperacillin–tazobactam, another β-lactam combination, was observed in 18.18% of isolates. This aligns with reports from Lebanon (Eid et al., 2025), where a 22% resistance rate was reported [22]. In contrast, higher resistance rates were recorded in studies from Qatar (69.5%) [23], whereas a study by Owaid and Al-Ouqaili (2025) reported no resistance to this compound [24]. These differences may be attributed to variations in clinical prescribing patterns, regional antibiotic use, and genetic determinants of resistance within local bacterial populations. Regarding aztreonam, a monobactam antibiotic, 34.55% of isolates exhibited resistance. This finding mirrors the results of a study by Salleh et al. (2025) conducted in Wasit Province, which reported a resistance rate of 34.5% [25]. However, it deviates from other regional studies, such as those by Alkhulaifi & Mohammed (2023), which documented resistance rates of 82.11% [26].

Moderate resistance to imipenem (30.91%) and meropenem (21.82%) was also noted. These results are consistent with prior findings by Mohammed et al. (2022), who reported resistance rates of approximately 30% to imipenem in Baghdad [27]. The meropenem resistance rate was close to the 26.1% reported by Yasen (2023) [28], and slightly lower than the 31.8% documented by Seenaa et al. (2024) [29]. As members of the carbapenem class, both imipenem and meropenem are highly potent against *P. aeruginosa*, owing to their excellent cell wall penetration and low susceptibility to hydrolysis by common β-lactamases. However, emerging resistance—possibly through metallo-β-lactamase production or porin loss—has raised concerns about their long-term efficacy [30]. The aminoglycoside tobramycin showed a resistance rate of 21.82%, which is in proximity to the 28.9% reported by Kamal et al. (2018) [31], but significantly lower than the 58.4% observed by Salih et al. (2022) [32]. This difference may be due to reduced clinical use of aminoglycosides in certain settings, thereby limiting selective pressure for resistance development. For fluoroquinolones, norfloxacin and levofloxacin showed resistance rates of 23.64% and 32.73%, respectively. The resistance to norfloxacin aligns with the 22.72% reported in Sulaymaniyah by Seenaa et al. (2024) [33], but differs markedly from the 71.75% found by Almuttairi and Abdulla (2023) [34]. Similarly, levofloxacin resistance in this study resembles the 35.5% reported in Syria by Shanan et al. (2025) [35], yet is significantly lower than the 100% resistance reported by Hameed et al. (2021) in Karbala [36]. Variation in fluoroquinolone resistance likely stems from differences in prescribing frequency and the presence or absence of plasmid-mediated quinolone resistance genes across regions.

Minimum Inhibitory Concentration (MIC) of Colistin

The minimum inhibitory concentration (MIC) of colistin against *P. aeruginosa* isolates was determined using the broth microdilution method in accordance with Clinical and Laboratory Standards Institute (CLSI, 2024) guidelines. The MIC for each isolate was defined as the lowest antibiotic concentration that completely inhibited visible bacterial growth. Isolates were classified as resistant if the MIC was ≥ 4 µg/mL and as susceptible if the MIC was ≤ 2 µg/mL, following the established CLSI breakpoints.

The analysis of colistin susceptibility among the 55 clinical isolates of *P. aeruginosa* revealed a highly alarming trend. Of the total isolates, 53 (96.36%) were resistant, exhibiting MIC values ≥ 4 µg/mL, whereas only 2 (3.64%) were susceptible, with MIC values below the CLSI-defined resistance threshold (2024). This resistance rate is significantly higher than that reported by Al-Ameen and Ghareeb, who documented a resistance rate of only 21.7% in their previous investigation [37]. Such a high prevalence of colistin resistance is of considerable clinical concern and may indicate a substantial reduction in the drug's efficacy in treating *P. aeruginosa* infections within the studied population.

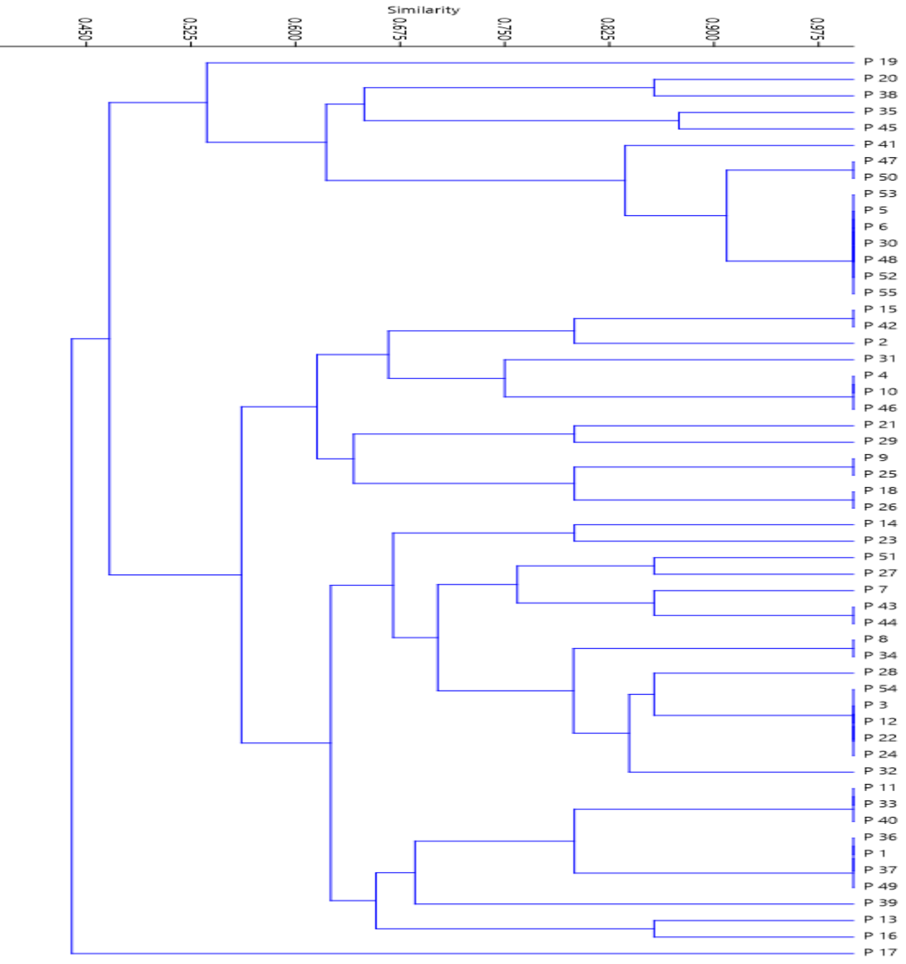
Specifically, colistin resistance was evident across nearly all sources, including isolates from urine, ear swabs, sputum, blood, wounds, and burn infections. The highest observed MIC values reached >1024 µg/mL, indicating an extremely high level of resistance. Other isolates also exhibited maximal resistance levels, suggesting the emergence of colistin-tolerant clones. Only two isolates were identified as colistin-sensitive, showing MIC values < 4 µg/mL. These exceptions underscore the rarity of susceptible strains in this dataset and suggest the potential impact of localized antimicrobial-use policies or reduced exposure to colistin among certain patients. The widespread resistance to colistin observed in this study is particularly concerning, given its role as a last-resort antibiotic for treating infections caused by multidrug-resistant *P. aeruginosa*. The high MIC values suggest reduced drug efficacy, likely associated with genetic mutations in regulatory systems such as pmrA/pmrB or phoP/phoQ, or structural modifications of lipid A in the outer membrane. This resistance pattern necessitates urgent review of treatment protocols and the introduction of alternative therapeutic strategies in healthcare settings where colistin resistance is rising. Additionally, factors such as biofilm formation, monotherapy, and selective pressure from prolonged colistin exposure may facilitate the development and persistence of resistant strains.

Colistin is generally considered effective against *P. aeruginosa*, particularly in strains exhibiting resistance to other major antibiotic classes such as carbapenems, fluoroquinolones, and aminoglycosides [38]. Its mechanism of action involves binding to lipid A components of the bacterial outer membrane, leading to membrane disruption and subsequent bacterial cell death [39]. The observed elevation in MIC values among clinical isolates strongly suggests a diminishing role for colistin as a last-resort therapeutic agent. This trend underscores the urgent need for revised treatment protocols and the implementation of comprehensive antimicrobial stewardship strategies to minimize further resistance development [40].

3.5. The dendrogram

The cluster analysis (dendrogram figure1) of the antibiotic susceptibility test results for the 55 isolates under scrutiny revealed 11 clones and 21 isolates. They were further divided into two major groups, group A and B (Figure 1), on which there was a similarity of 44% between the two groups. Group A was composed of isolates (17) (otitis) resistant to the antibiotics (LEV, PTZ), and Group B was composed of two groups. B1 and B2. Group B1 contained 2 clones and 6 isolates and could be further subdivided into two parts. In the initial portion, isolate (19) (otitis) was resistant to (COLISTIN, NOR, TTC, and PRL). With regard to the second section, it was additionally split in two. The initial component had isolates (20, 38) that were resistant to the antibiotics (COLISTIN, ATM, TTC, and CFE). The second part consisted of isolates (35, 45) and were resistant to (COLISTIN, MRP, IMI, TTC, and CFE). The second section contained isolates (41, 47, 50, 53, 5, 6, 30, 48, 52, 55) and was, in turn, split into 2 sections. The first segment consisted of isolates (41) (wounds) showing a resistance to the antibiotics (COLISTIN, LEV, NOR, TOB, ATM, TTC, CFE, and PRL). The second one encompassed two clones. The first clone (47, 50) (burn wound infection) revealed resistance to the following antibiotics: COL, LEV, NOR, TOB, MRP, ATM, TTC, CFE, and PRL. The second clone included isolates (53, 5, 6, 30, 48, 52, and 55). The first isolated strain was (burn, urine, sputum, burn, burn, and burn). This clone was resistant to all the antibiotics used. B2 was divided into two subgroups. The initial subgroup was divided into two parts. On the first part, isolates (15, 42) on (wound swabs, and otitis swabs) did not respond to antibiotics COL, TTC, and CFE, and isolate (2) was resistant to COL, IMI, TTC, and CFE. Isolate (31) was resistant to COL, TTC, CFE, and PRL. revealed that clone (4, 10, 46) isolated from (urine, otitis and burns) were resistant to (COL and TTC). The second included isolates (21, 29), both of which were resistant to (COL, IMI, and TTC). The two clones included the first clone (9, 25) (otitis), both resistant to the antibiotics (COL, IMI, and TTC), and the second clone (18, 26) (otitis), both resistant to (COL, LEV, and TTC). The second group B2 had two sections. Isolates (14, 23) resistant to the TTC antibiotic were included in the first section. The second section started with isolates (51, 27) that are alike in resisting (COL and IMI). The second section comprised isolate (7) and clone (43, 44) (wound swabs) and were resistant to (COL, IMI, TTC, and CFE). The second section. The clone represented in the first column consisted of the isolates (8, 34), with both plates being (otitis and blood), and each being resistant to (COL and TTC). Isolate (28) was resistant to (COL, NOR, TTC, and CFE). The remaining isolates (54, 3, 12, 22, 24) were (burns, urine, otitis, otitis, and otitis) respectively. All five isolates were resistant to (TTC, COL, and CFE) as well. Isolate (32) was resistant to (COL, TTC, and CFE). The second group was divided into two sections. In section one, the first clone (11, 33, 40) isolates of (otitis, sputum, and wounds), which were resistant to (COL, TTC, and CFE).

A second clone (1, 37, 49) was (urine, blood, burns) showing resistant to (COL, TTC and CFE). Isolate (39) was resistant to (COL and TTC).The second section, contained isolates (13, 16) that were resistant to (COL, TTC, CFE and PRL).



Figure(1): The Dendrogram

3.6. Multidrug Resistance Patterns (MDR and XDR)

Out of the 55 *P. aeruginosa* isolates analyzed in this study, 48 isolates (87%) exhibited a multidrug-resistant (MDR) phenotype, showing resistance to three to eleven different antibiotics, as detailed in Table 3. This prevalence is consistent with findings reported by Ridha (2023) in Diyala Province, which documented an MDR rate of 82% [41], but contrasts significantly with the lower rate of 46% observed in a study by Abdallah and Gabur (2021) in Nasiriyah [42]. Additionally, 10 isolates (18%) were categorized as extensively drug-resistant (XDR), indicating resistance to nearly all available antibiotic classes tested. The high incidence of both MDR and XDR strains highlights a growing concern for public health, as such resistance profiles severely limit treatment options and are often associated with increased morbidity and mortality.

In recent years, the global rise of MDR and XDR *P. aeruginosa* has been driven by several factors, including the irrational and excessive use of antibiotics, lack of effective antimicrobial stewardship, and the spread of resistance genes through horizontal gene transfer. Moreover, the absence of robust surveillance systems and well-defined therapeutic guidelines in many regions further exacerbates this issue. These resistant strains often harbor multiple resistance mechanisms, such as β-lactamase production, loss of outer membrane proteins, efflux pump overexpression, and target-site modifications. Based on the extent of resistance, isolates are classified into MDR (resistant to ≥3 antimicrobial classes), XDR (resistant to all but one or two classes), and, in rare cases, pandrug-resistant (PDR) strains—those resistant to all available agents.

Infections caused by such pathogens are frequently associated with higher treatment failure rates and limited therapeutic options, underscoring the urgent need for controlled antibiotic use and continuous resistance monitoring.

Table (3): Classification of bacterial resistance

Resistance type	No. of isolates	Percentage
MDR	38	69%
XDR	10	18%
Total	48	87%

3.7. Pigment Production

The production of pyocyanin pigment by the *P. aeruginosa* isolates was evaluated by streaking cultures onto King A and King B agar (Figure 5). All 24 selected isolates were tested for pigment production on King A agar, resulting in a 100% detection rate. This finding is consistent with the results of Daher (2024), who reported a comparable positivity rate of 96% [43]. No pigment production was observed on King B agar, likely due to its low phosphate concentration, which inhibits pigment biosynthesis. In contrast, King A medium contains elevated levels of magnesium and potassium, known to stimulate pyocyanin synthesis [44].

Pyocyanin is considered one of the key virulence factors of *P. aeruginosa*. It plays a critical role in biofilm formation, facilitates microbial competition by suppressing the growth of rival strains, and contributes to host tissue damage through its pro-inflammatory and cytotoxic effects [45]. The universal presence of pyocyanin production among the isolates studied reinforces the aggressive pathogenic potential of *P. aeruginosa* in clinical settings.

4. CONCLUSION

The present study highlights the high prevalence of *P. aeruginosa* in clinical infections, especially those involving the ear, burns, and wounds. The isolates demonstrated considerable multidrug resistance, with colistin resistance emerging as a particularly alarming trend. The presence of multiple virulence factors, including hemolysin and pyocyanin, further underscores the clinical significance of this pathogen. These findings emphasize the urgent need for enhanced infection control practices, routine antimicrobial surveillance, and stricter antibiotic stewardship to mitigate the spread of resistant *P. aeruginosa* strains in healthcare settings.

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