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Antibiotic Susceptibility, Biofilm Formation, and Phylogenetics of *Pseudomonas aeruginosa* in Cystic Fibrosis Patients

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ABSTRACT

Background and Aim: *Pseudomonas (P.) aeruginosa* is a Gram-negative bacterium that causes lung infection in cystic fibrosis (CF) patients by forming biofilms, which contribute to antibiotic resistance. The genetic diversity among strains could be important epidemiologically in the community of CF patients. This study investigated *P. aeruginosa* strains from CF patients' sputum, focusing on their antibiotic resistance, biofilm production, and genetic diversity to understand their epidemiological relations.

Materials and Methods: Sixty-five samples of *P. aeruginosa* were isolated from CF patients with pulmonary infection symptoms (October 2023 to October 2024) admitted to Imam Khomeini Children's Hospital in Tehran. The antibiotic susceptibility patterns of these strains were examined using disk diffusion method (Kirby-Bauer), based on the CLSI standard. Moreover, to evaluate colistin susceptibility, broth microdilution (BMD) method was used. The strains' ability to produce biofilms was evaluated using a microtiter plate (MTP) assay. Finally, RAPD-PCR (Random Amplified Polymorphic DNA-Polymerase Chain Reaction) was employed for genetic analysis, using the short primer 272.

Results: About 61.53% of *P. aeruginosa* strains exhibited multidrug resistance (MDR), while 30.7% of the strains exhibited extensively drug resistance (XDR) characteristics. The highest levels of antibiotic resistance were found against amoxicillin, amikacin, and cefepime. XDR and MDR strains exhibited strong biofilm-forming ability. The 65 studied strains were classified into 19 groups based on RAPD-PCR patterns, with six strains sharing similar genetic traits divided into four categories.

Conclusion: The study showed elevation in antibiotic resistance in *P. aerug*inosa and established a strong connection between biofilm production and resistance. Some isolates exhibited similar genetic patterns, indicating possible cross-infection.

Keywords: Biofilm, Cystic Fibrosis, Multidrug Resistance, RAPD-PCR, *Pseudomonas aeruginosa*

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1. Introduction

Cystic fibrosis is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which is located on the long arm of chromosome 7 **(1, 2)**. The CFTR gene was identified in 1989. It encodes a transmembrane protein that plays a crucial role in ion transportation in epithelial cells. The CFTR protein functions as a chloride channel. In CF patients, secretions from the lungs, pancreas, intestines, and reproductive organs become thick and sticky. Additionally, the salt concentration in sweat gland secretions increases, resulting in the loss of the body's necessary salt through sweat **(3, 4)**.

Chronic lung infections caused by *Pseudomonas (P.) aeruginosa* in CF patients are one of the most crucial reasons for mortality and morbidity in this population **(5, 6)**.

CF patients are initially infected with environmental strains of *P. aeruginosa*. These strains undergo both phenotypic and genetic changes within the lungs of these patients. By producing biofilms and adapting to the lung environment, they cause chronic and persistent infections. This infection has the potential to kill the patient if they are not treated **(7)**. The lung infections caused by *P. aeruginosa* in CF patients are linked to the existence of mucoid strains that produce alginate and form biofilms. A biofilm is a structured community of bacteria organized within a polymeric matrix created by these bacteria. This matrix consists of polysaccharides, proteins, and extracellular DNA. In the lungs of CF patients, alginate is the primary component of the matrix produced by *P. aeruginosa* **(8, 9)**.

Biofilms contribute to chronic infections because bacteria in these environments show greater resistance to antibiotics and evade phagocytosis, along with other components of the innate and adaptive immune systems. As a result, a strong antibody response is generated, leading to chronic inflammation mediated by immune complexes produced by polymorphonuclear leukocytes. This persistent inflammation is primary reason for the lung tissue damage in CF patients. The development of biofilms in the lungs of CF patients is linked to increased mutation rates, slow bacterial growth, and the bacteria adaptation to the lung environment and antibiotic treatments. Furthermore, low metabolic activity of bacteria and their extended cell doubling time in CF lungs contribute to their resistance to antibiotics **(10)**. Common resistance mechanisms in bacteria, such as chromosomal beta-lactamases, efflux pumps, and mutations in antibiotic target molecules, contribute to the persistence of biofilms. Biofilm formation can be prevented by implementing preventive measures and adjusting initial antibiotic treatments **(11)**. Therefore, using appropriate antibiotics and implementing therapeutic methods and prophylaxis are important in the treatment of CF patients **(12)**. Assessing the antibiotic susceptibility patterns of bacteria and examining the capability of *P. aeruginosa* strains to form biofilms can aid in identifying current bacterial resistance within a sample population. This finding is a crucial step toward ensuring the effectiveness of antibiotics in CF patients' treatment.

Pseudomonas aeruginosa showcases a remarkably diverse and variable genome, a feature that empowers this bacterium to thrive in fluctuating environmental conditions and adapt to various hosts. This genetic richness allows it to display an impressive array of phenotypes. Consequently, relying solely on phenotypic tests for identification and research is inadequate; robust molecular methods must also be employed. Since CF patients are highly susceptible to chronic lung infections, identifying and characterizing the genetic makeup of infecting strains can provide valuable insights into the infection sources, spread patterns, and antibiotic resistance profiles.

Genetic investigation of the strains infecting CF patients is essential not only for understanding the pathogenic characteristics of the microbes involved but also for epidemiological purposes. It aids in tracking transmission routes, preventing outbreaks, managing antibiotic resistance, and ultimately improving the care and prognosis of CF patients. Among various molecular techniques, RAPD-PCR stands out as a highly reliable approach, celebrated for its precision in classifying strains and uncovering their genetic relationships. In Iran, where the diversity of Pseudomonas species is abundant and their distribution is widespread, there is a pressing requirement to explore the genetic similarities among *P. aeruginosa* isolates, particularly those from CF patients. Therefore, embarking on this study is not only necessary but also crucial for advancing our understanding of this adaptable bacterium **(13, 14)**.

This study aimed to explore the fascinating relationship between the genetic profiles of *P. aeruginosa* strains and their patterns of antibiotic susceptibility and biofilm production. By isolating these strains from the sputum of CF patients, we seek to shed light on essential aspects of infection management for this particular vulnerable population.

2. Materials and Methods

2.1 Bacteria Isolation

In this study, 65 samples of *P. aeruginosa* were collected from the sputum of patients admitted to Imam Khomeini Children's Hospital in Tehran (October 2023 to October 2024) at the time of admission. The collected samples were cultured on blood agar medium. The morphology of the bacterial colonies was evaluated based on their mucoid and non-mucoid characteristics, and the isolated bacteria were identified using standard biochemical tests.

2.2 Bacteria Identification

Standard bacteriological methods were employed to identify the strains of *P. aeruginosa* through colony morphology, hemolysis, Gram staining, cytochrome oxidase testing, catalase testing, and pyocyanin pigment production. Additionally, biochemical reactions in diagnostic culture media such as OF, SIM, KIA, LIA, and TSI were utilized to confirm the presence of *P. aeruginosa* strains **(15, 16)**. To preserve the strains for further experiments, they were stored in TSB medium with 15% glycerol at -20°C. It should be noted that all reagents and the media were manufactured by Indian company (Himedia) **(17, 18)**.

2.3 Antimicrobial Susceptibility Test

Antibiotic susceptibility patterns of the isolated strains were examined using Mueller-Hinton agar test and disk diffusion technique (Kirby-Bauer) according to the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) **(18, 19)**. In addition, the quality control strain used in this study was *P. aeruginosa* ATCC 27853. Six different classes of antibiotics, including cephalosporins (ceftazidime, cefotaxime, and cefepime), aminoglycosides (amikacin), carbapenems (Imipenem, meropenem), fluoroquinolones (ciprofloxacin), beta-lactams (amoxicillin, ticarcillin, piperacillin), penicillins (piperacillin and tazobactam), polymyxins (colistin) were used to investigate the susceptibility to these antibiotics.

Although the disk diffusion method is commonly used in clinical laboratories, it is considered an unreliable test for colistin due to the ineffective diffusion of colistin into the agar. This poor diffusion is caused by the electrostatic interactions between colistin and acid or sulfate groups present in the agar, which leads to smaller inhibition zones. Thus, in this study broth microdilution (BMD) method was used to check the susceptibility pattern of colistin. Moreover, non-susceptible *P. aeruginosa* isolates to at least one agent in ≥ 3 and ≥ 6 different antimicrobial categories were defined as MDR and XDR, respectively **(15, 20)**.

2.4 MIC Determination for Colistin Antibiotic

Resistance to colistin was determined phenotypically by BMD method, using colistin sulfate powder (Sigma) according to the CLSI guidelines. Cation-adjusted Mueller-Hinton broth (CaMHB) was used for the routine susceptibility tests of Gram-negative bacteria. The final pH was adjusted between 7.2-7.4. MIC was performed with a range of 0.25-256 µg/mL, in which MIC ≤ 2 was considered semi-susceptible and MIC ≥ 2 was considered resistant.

The test results were repeated three times to ensure reproducibility. The standard strain *P. aeruginosa* ATCC27853 was used as the standard strain for evaluating the quality control of diagnostic tests and antibiograms **(21, 22)**.

2.5 Biofilm Formation Assay

First, 5 ml of TSB medium (Merck, Germany) supplemented with 1% glucose was used to inoculate the isolates, then the isolates were incubated for 24 hr at 37 °C. Subsequently, to create a suspension of 10^6 CFU/mL, the bacterial culture was diluted 1/100 using a fresh, sterile TSB medium. Afterward, 200 µL of diluted suspension was distributed into each well of a polystyrene 96-well microtiter plate (Jetbiofil, Canada). In the next step, the wells were rinsed three times with 200 µL sterile phosphate-buffered saline (PBS) (pH 7.4). After fixing the biofilms for 15 min with absolute methanol (Merck, Germany), 200 µL of crystal violet solution (1.5% w/v) (Merck, Germany) was added to each well. The unbound stain was disposed of, and 150 µL of acetic acid (33 % v/v) (Merck, Germany) was added to the stained biofilms for 20 min.

Ultimately, the optical density (OD) for every sample was measured using a microplate ELISA reader (Biotek, USA) set at 620 nm and finally assessed against the baseline OD value (ODc). The ODc was defined as three standard deviations above the mean absorbance of the negative control, with the modified TSB medium (plus 1% glucose) acting as the negative control. Using the following formulas, the isolates biofilm formation pattern was divided into four groups: OD < ODc (non-biofilm producer), ODc < OD < 2xODc (weak biofilm producer), 2xODc < OD < 4xODc (moderate biofilm producer), 4xODc < OD (strong biofilm producer). All assays were conducted in triplicate, with *P. aeruginosa* ATCC 27853 serving as the positive control **(23)**.

2.6 PCR Assay Design and Optimization

a) DNA Extraction

DNA extraction was carried out using the boiling technique. Concisely, 1.5 mlLof *P. aeruginosa* culture, which was inoculated into TSB medium was added to

500 µL distilled water. Then, *P. aeruginosa* was decomposed by boiling for about 10 min and centrifuged. Subsequently, the supernatants were applied to PCR to detect biofilm-forming genes, and the samples were kept at −20°C as DNA templates **(24)**.

b) RAPD-PCR

Primer 272, 5'AGCGGGCCAA3', is a specific primer chosen for *P. aeruginosa* fingerprinting, aimed at evaluating the phylogenetics of the strains isolated from the sputum of CF patients. The RAPD-PCR reaction mixtures (25 µL), were optimized for detecting *P. aeruginosa* and included the following components: 40 ng of genomic DNA, 50 mM of oligonucleotide primer (Cinnagen), 1 unit of Taq DNA polymerase (Cinnagen), 250 µM of each deoxynucleoside triphosphates (Cinnagen), 10 mM Tris-HCl buffer at pH 8.0, 50 mM potassium chloride (KCl), 0.001% gelatin, and 3 mM magnesium chloride ($MgCl_2$).

Finally, 25 µL of mineral oil was added to each reaction mixture, and RAPD-PCR process was conducted using the Eppendorf AG apparatus according to the specified thermal cycling conditions, including; (i) 4 cycles of 5 min each at 94°C, 36°C, and 72°C; and (ii) 30 cycles of 1 min each at 94°C, 36°C, and 2 min at 72°C, subsequently final extension step at 72°C for 10 min.

One-third of each mixture was loaded onto 1.5% agarose gel and run in 1x TBE buffer at a voltage of 9 V/cm for 3 hr. Molecular size standard, specifically a 100 bp-3 Kbp ladder (Cinnagen), was included in the gel. The gels were stained with ethidium bromide and photographed using a Gel document (UVP) device. To check the results by RAPD-PCR pattern obtained from the relevant strains, a dendrogram was created using the UPGMA method **(14, 25)**.

2.7 Detection of virulence genes

Statistical analysis was conducted using SPSS 22 software to classify various parameters. To summarize the prevalence or frequency of MDR, XDR, and biofilm production within the sample groups, counts, percentages, means, and standard deviations were used. Chi-Square Test and Fisher's Exact Test were employed to assess whether there is a significant association between RAPD-PCR clustering patterns (e.g., genotypes) and resistance categories (MDR, XDR) or biofilm production status. A P-value ≤ 0.05 was considered statistically significant.

3. Results

3.1 Antimicrobial Susceptibility Test Results

Among 65 strains, 61.53% were MDR, while 30.7% were XDR. The highest resistance rates were observed for amoxicillin (96.92%), amikacin (95.38%), and cefepime (93.84%). In contrast, the highest susceptibility rates were observed for colistin (92.3%), meropenem (84.61%), and ciprofloxacin (81.53%).

Additionally, 85% of the isolates were resistant to more than five antibiotics. The results of antibiotic susceptibility among the strains are illustrated in Figure 1. Notably, the prevalence of MDR and XDR strains increased with age.

3.2 Biofilm Production Assay Results

According to the results of *P. aeruginosa* strains capacity to form biofilm, XDR strains showed a significant capacity for robust biofilm production, with 85% of these strains exhibiting biofilm formation (Table 1). It is noteworthy that 82% of MDR strains and 85% of XDR strains were able to form robust biofilms.

In contrast, none of the non-MDR and non-XDR strains were able to form strong biofilms; however, 60% of these strains formed weak biofilms. The results indicate that biofilm production significantly contributes to development of MDR and XDR strains.

It is worth noting that some strains exhibited MDR or XDR antibiotic resistance patterns but produced weak or moderate biofilms. After analyzing the biofilm production by MDR and XDR strains, we found that 5% of these strains formed weak biofilms. In addition, 12.5% of MDR strains and 10% of XDR strains formed moderate biofilms. The observed results indicate that MDR strains, which exhibit weak to moderate biofilm formation, may employ various additional resistance mechanisms alongside their biofilm formation to withstand antibiotics.

One of the most remarkable findings from the results is that MDR and XDR strains capable of producing strong biofilms were more frequently observed in older patients. In contrast, strains that produced weak biofilms were more common among younger patients.

3.3 RAPD-PCR Results

Various molecular techniques are available for the study of bacterial genetic diversity and phylogenetic classification. One of the most widely used methods is RAPD-PCR, which provides a reliable and straightforward approach for the bacterial differentiation assays **(26, 27)**.

Based on RAPD-PCR patterns obtained from *P. aeruginosa* strains, a dendrogram was created using the UPGMA method (Figure 2). The studied strains could be classified into 19 groups according to this pattern. Table 2 illustrates the classification of *P. aeruginosa* strains into various cluster groups based on the obtained dendrogram. According to the dendrogram, 6 bacterial

strains with similar genetic patterns were grouped into 4 distinct categories.

In groups B, D, N, and Q, 6 strains displayed similar genetic patterns. It is important to note that while these strains exhibited relatively comparable antibiotic sensitivity and biofilm production patterns, the results were not entirely identical. Nevertheless, the strains demonstrated a high degree of similarity in their genetic patterns.

A significant correlation was detected between the presence of MDR, XDR and strong biofilm producer strains within the same branch of RAPD-PCR dendrogram (*P*=0.023).

Table 1. Correlation between biofilm formation capacity and resistance phenotypes in *P. aeruginosa* isolates.

Type of resistant strains	Strong-biofilm producer	Moderate-biofilm producer	Weak-biofilm producer
MDR	82.5%	12.5%	5%
XDR	85%	10%	5%
Non-MDR & Non-XDR	0	40%	60%

Significant correlations were observed between the ability of MDR and XDR strains to produce strong biofilms (*P*=0.004 and *P*=0.003, respectively).

Table 2. Dendrogram-based classification of *P. aeruginosa* into different clusters.

Cluster groups	Number of strains
A	58,26,22,23
B	54,35,36,53,32,33
C	28,27,24,25
D	19,20,10,11,15,16
E	42,43
F	40,41
G	29,38
H	65,46,47,44,45
I	51,56,62,64
J	31
K	13

L	1,4
M	34,37,21
N	39,49,57,63,55,60
O	12
P	7,8,17
Q	59,61,30,48,50,52
R	6,5,3,2
S	9,14,13

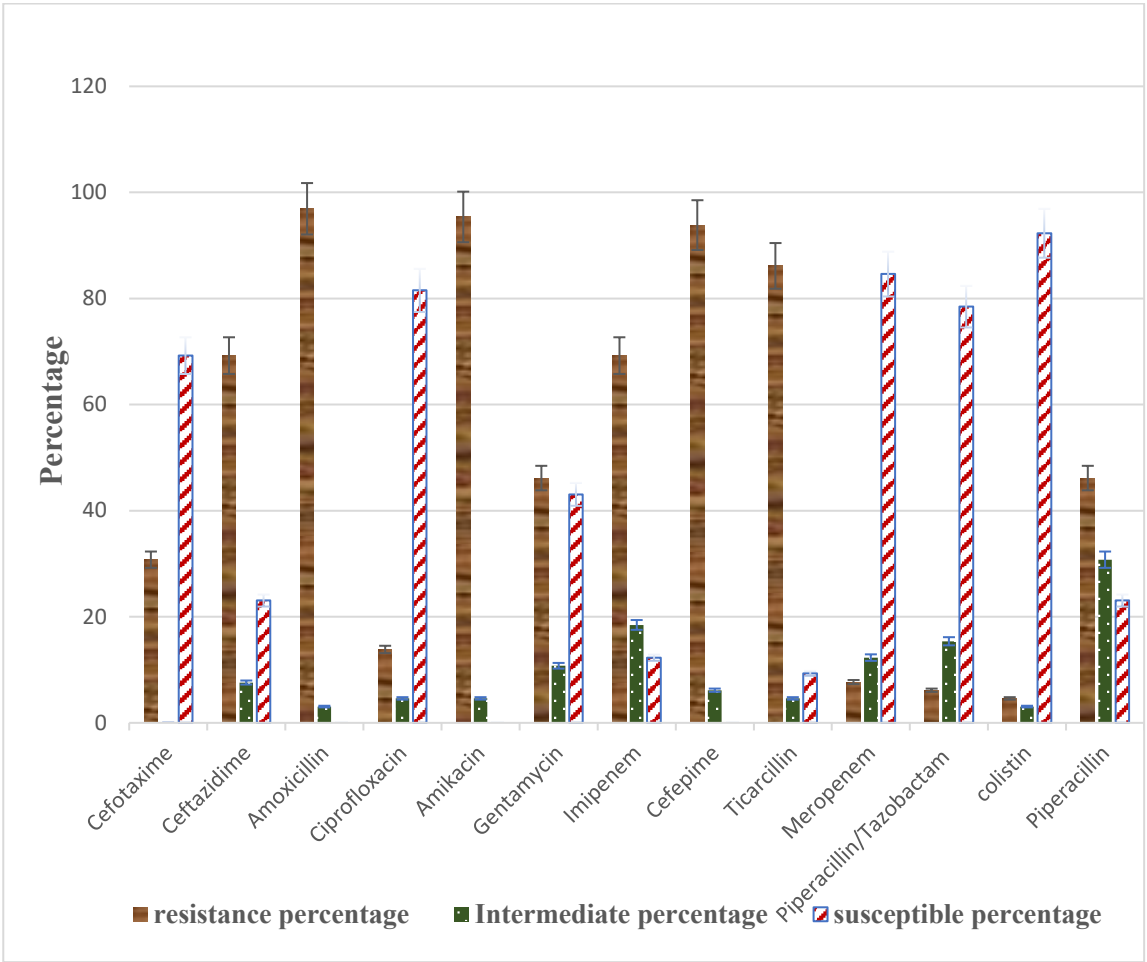

Figure 1. Antimicrobial resistance pattern in isolated strains: The bar graph illustrates the trend of antimicrobial resistance in isolated strains. The horizontal axis represents the types of antibiotics, while the vertical axis indicates the rates of susceptibility and resistance as percentages **(Designed by Authors, 2025)**.

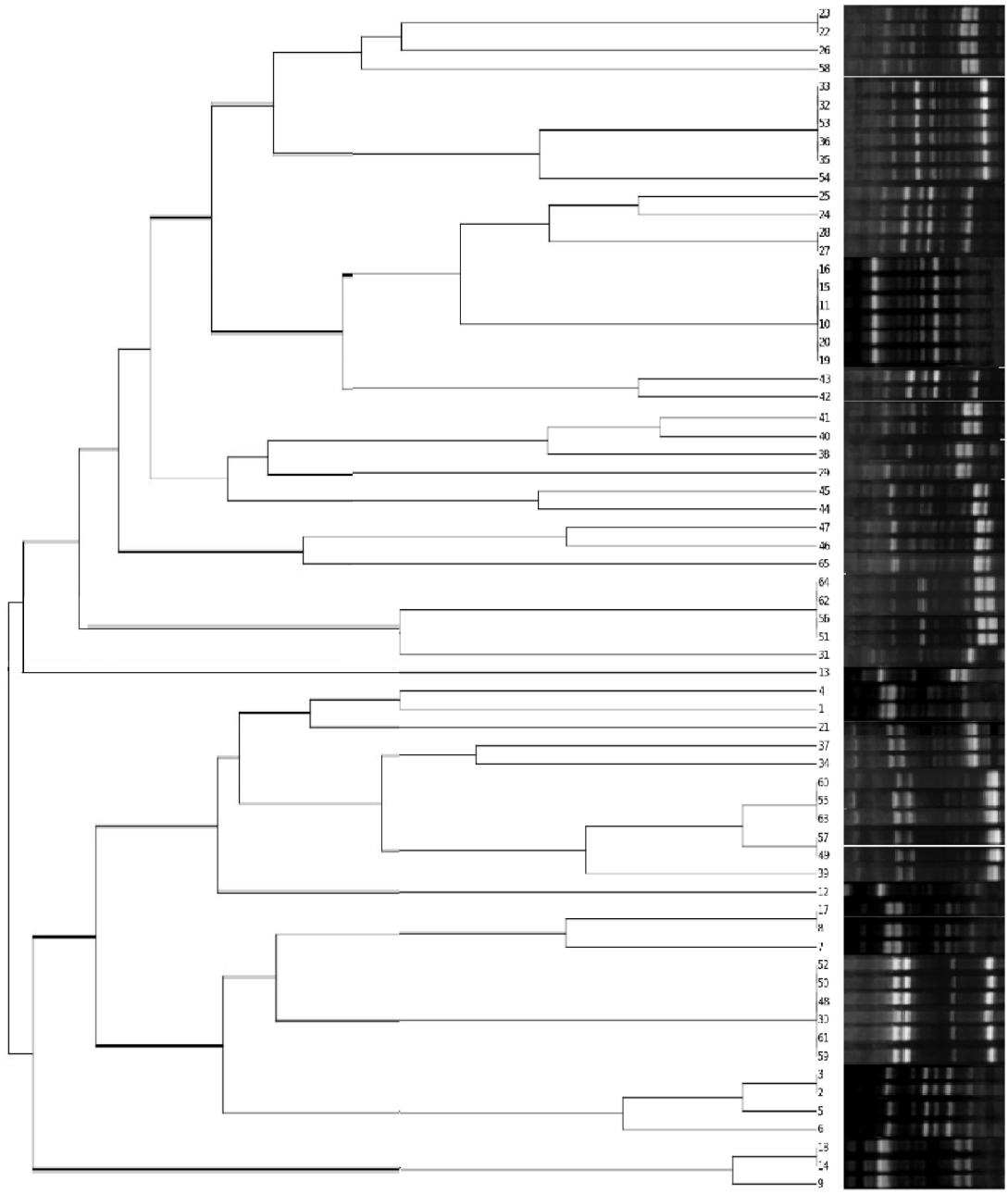

Figure 2. Dendrogram of *P. aeruginosa* strains showing genetic diversity via RAPD-PCR with primer 272. The image on the right displays the gel electrophoresis of RAPD-PCR, while the related dendrogram is presented on the left **(Designed by Authors, 2025)**.

4. Discussion

In this study, 65 strains of *P. aeruginosa* were isolated from the sputum of CF patients referred to Imam Khomeini Hospital in Tehran.The antibiotic susceptibility and biofilm production ability of the strains were evaluated. The results indicated that MDR and XDR strains exhibited greater capacity for the strong biofilm production. RAPD-PCR was employed to analyze the genetic and epidemiological distribution of the strains within the patient population. Several isolates from patients displayed high genetic similarity, which may be attributed to cross-contamination among patients.

It is essential to ensure prompt and effective treatment for *P. aeruginosa* in CF patients. Examining the antibiotic susceptibility patterns of this bacterium in the specific population of CF patients can provide valuable insights into trends in antibiotic resistance. This information can help physicians choose effective treatment options, especially after detecting Pseudomonas infections in the lungs of CF individuals.

Rastegar-Kashkouli et al **(25)** demonstrated that antibiotics colistin and ticarcillin are among the most effective treatments for *P. aeruginosa* in CF patients. The findings of this study align with previous results regarding colistin; however, it revealed a high level of resistance to ticarcillin.

In a study conducted by Raoofi et al **(28)** at Sina Hospital, researchers evaluated the microbiological susceptibility and the potential for biofilm formation in 60 clinical strains of *P. aeruginosa* using microplate method. The results indicated that strains with multiple antibiotic resistance produced significantly strong biofilm, which may be consistent with the findings of the current study **(28)**.

In addition, Ramazani et al **(29)** reported that 62.4% of *P. aeruginosa* strains were capable of producing biofilms by showing resistant to ceftazidime, which aligns with the resistance rate found in our current study. However, the biofilm production in our research was higher (76%). This increase is likely attributed to the number of samples **(29)**.

In a study by Karamollahi et al **(30)** among 113 tested strains of *P. aerugi*nosa exhibited strong biofilm formation, 40 strains showed the highest resistance to cefotaxime, meropenem, imipenem, and piperacillin. The resistance rates were low for cefotaxime and meropenem; however, resistance to imipenem and piperacillin were 69.23%, and 46.15%, respectively, which aligns with the findings of Karamollahi et al **(30)**. All observed differences in resistance rates may be attributed to variations in sample size, infection site, climate, and patient conditions.

Hassanzad et al **(31)** evaluated the trend of antibiotic resistance in CF patients at Masih Daneshvari Hospital from 2014 to 2019. They found that *P. aeruginosa* exhibited the highest susceptibility to colistin (93.3%) and ceftazidime (65.2%).

The results of the current study regarding two antibiotics; colistin and ceftazidime, are consistent with those in previous research. This study found that *P. aeruginosa* exhibited high resistance to several antibiotics, including amoxicillin, aminoglycosides (such as amikacin), cefepime, ticarcillin, imipenem, and ceftazidime. These findings align with multiple reports and evaluations from Iran, which have also shown that *P. aeruginosa* isolates in our region demonstrate resistance to a wide range of antimicrobial agents **(32, 33)**.

This study showed that while more than half of the *P. aeruginosa* strains exhibit high resistance to five classes of antibiotics, some antibiotics continue to be effective against these strains. The identified effective antibiotics include colistin, meropenem, ciprofloxacin, piperacillin/ tazobactam, and cefotaxime.

We indicated that older patients are more likely to be infected with mucoid and XDR strains. This increase in likelihood may be due to two possible factors: Elderly patients may have been initially infected with mucoid or XDR strains at the onset of their illness. Alternatively, non-mucoid and MDR strains present in their bodies could have evolved into mucoid and XDR strains over time. In younger patients, resistant strains may arise from hospital and clinic environments, which could serve as sources of infection or cross-infection among CF patients **(33)**.

The high prevalence of multiple antibiotic resistances in *P. aeruginosa* strains, particularly those isolated from CF patients presents a significant risk to the health and lives of these individuals as well as to the broader community **(34)**. Therefore, it is crucial to research antibiotic resistance in the CF community. This investigation can help identify effective treatments, develop appropriate management strategies, and uncover new antibiotic targets for the chronic *P. aeruginos*a infections in CF patients.

The current findings reveal a significant prevalence of MDR/XDR isolates in our country, emphasizing the crucial requirement for comprehensive microbiological surveillance, proper antibiotic prescribing practices, and strict adherence to antimicrobial policies within the healthcare systems.

One significant reason for the persistence and emergence of resistant strains of *P. aeruginosa* in the lung of CF patients is their ability to form biofilms. These biofilms can increase the risk of mortality in the affected individuals. Therefore, identifying strains that produce robust biofilms is essential for the effective treatment and prevention of chronic diseases in cystic fibrosis patients.

In this study, XDR and MDR strains have demonstrated a significant ability to produce strong biofilms. In contrast, none of the strains that showed non-multidrug resistance were able to produce strong biofilms, and 60% of these strains produced weak biofilms. The findings revealed that biofilm production plays a significant role in the development of multidrug resistance.

Kabir et al **(33)** reported that the strains capable of producing biofilms exhibit much higher level of antibiotic resistance compared to the strains that did not participate in biofilm formation even in the planktonic (free-floating) state. It is important to note that theier results were in accordance with the results of our study.

On the other hand, in this study, we observed that certain MDR strains, while exhibiting multiple resistances, demonstrated the ability to form biofilms at moderate levels. These results indicate that these

bacteria likely rely on alternative mechanisms of antibiotic resistance.

A study conducted by Heidari et al **(35)** evaluated antibiotic resistance, biofilm formation, and genetic diversity among 115 strains of *P. aeruginosa* isolated from various nosocomial infections. The results indicated high resistance rate to amikacin (70.6%), and strong sensitivity to colistin (96.1%). These findings were consistent with the results of the current study. More than half of the strains exhibited resistance to carbapenem, which is comparable to the resistance observed in isolated strains in this study. Additionally, most strains in their study showed resistance pattern consistent with MDR or XDR profiles. Furthermore, a 95% rate of biofilm formation was noted, closely aligning with the results of the current study. The strain typing identified 34 different strains, which were grouped into 3 main categories. Their findings indicated a direct correlation between resistance, strong biofilm formation, and the genetic diversity of the strains, which is consistent with the results of our study.

Multiple mechanisms are involved for antibiotic resistance in *P. aeruginosa* strains, not just biofilm formation. These mechanisms include reduced outer membrane permeability, the action of efflux pumps, specialized enzymatic activity, horizontal gene transfer for resistance, and mutations. Therefore, while biofilm formation is a critical factor, it is not the sole reason for high levels of antibiotic resistance observed. In particular, biofilm production significantly contributes to the chronic nature of Pseudomonas infections in CF patients **(28)**.

Traditional typing methods seem to be not suitable for identifying *P. aeruginosa* strains in CF patients. Therefore, molecular genetic techniques are used to investigate the phylogeny of this bacterium. In this study, we examined the phylogeny of the isolated bacteria using RAPD-PCR method, specifically with primer 272. RAPD-PCR stands out among molecular methods due to its speed, low cost, and high sensitivity.

Upon examining the dendrogram, 19 distinct groups of strains were identified. The number of bacteria within these groups varied, with each group containing between 1 and 6 strains. Out of the 19 groups, 4 contained strains with identical genetic patterns. However, when the strains were evaluated for antibiotic susceptibility and their ability to produce biofilm, it became evident that, despite genetic similarities, the strains were distinct from one another.

Based on dendrogram, strains categorized into groups B, D, H, and S displayed XDR pattern, demonstrating significant resistance to a range of antibiotics, including amoxicillin, amikacin, ticarcillin, cefepime, and imipenem. Additionally, strains in groups N, I, Q, C, R, and A exhibited MDR pattern, characterized by significant resistance to amoxicillin, amikacin, and cefepime. This distinction emphasizes the urgent requirement for targeted therapeutic strategies to address these challenging bacterial strains.

Additionally, strains belonging to groups B, D, H, and S demonstrated a strong ability to produce biofilms. In contrast, strains in groups N, I, Q, C, R, and A exhibited the capacity to produce both strong and moderate biofilms.

The reason for strains similarity is the potential for cross-infection in patients, as patients are exposed to resistant strains after clinic visits. Therefore, it would not be unreasonable to observe similar strains. Therefore, patients are advised to visit the medical centres only in case of need and emergency, and when present at medical centres, preferably wear mask and minimise their time in the clinics.

5. Conclusion

This study highlights significant public health and biosecurity concerns associated with APEC in broiler farms in Qazvin, Iran, evidenced by high rates of multidrug resistance (82%) and ESBL production (30%). Frequent detection of virulence genes, particularly *iss* (66%), combined with widespread biofilm formation, indicates a strong potential for environmental persistence and zoonotic transmission. Based on the demonstrated *in vitro* efficacy of commonly used disinfectants such as formaldehyde and hydrogen peroxide, we recommend routine rotation of disinfectants to prevent reduced effectiveness due to microbial adaptation. Additionally, the prudent use of antimicrobials, including limiting colistin use in veterinary practice, is essential to preserve the efficacy of critically important antibiotics. To mitigate the spread of resistant and virulent APEC strains, enhanced surveillance, strengthened biosecurity, and implementation of antibiotic stewardship programs are urgently needed in poultry production systems.

6. Declarations

6.1 Acknowledgment

The authors would like to express their gratitude and appreciation to the CF Iran Foundation.

6.2 Ethical Considerations

This research has been reviewed under the thesis title in Shahid Beheshti University and has been

approved by the Ethics Committee guidelines of Shahid Beheshti University with the ethics code (Ethical Number: IR.SBU.REC.1404.004).

6.3 Authors' Contributions

AE and NS designed the experiments. MME assisted with sample collection. AE performed the experiments, gathered data, and discussed the results and strategy. NS directed and managed the study. All authors have read and approved the final manuscript.

6.4 Conflict of Interests

No conflict of interest was found between the authors of this Article.

6.5 Financial Support and Sponsorship

This article is a part of a Doctoral Thesis in Microbiology, which has received formal approval from Shahid Beheshti University. Remarkably, it was undertaken without any external financial support, and all associated project costs were covered by the student.

6.6 Using Artificial Intelligence Tools (AI Tools)

The authors did not utilize AI Tools. Furthermore, all images and results presented herein were created through our efforts, ensuring authenticity and originality in every aspect of our work.

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