Original Article

Distribution of *toxA*, *algD*, *rh1I*, *pilA*, and *pilB* Genes among Extensively Drug-Resistant *Pseudomonas aeruginosa* Isolated from Burn Patients in Al-Diwaniyah City, Iraq

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Abstract

Background: One of the most common pathogenic organisms invading burn sites and causing severe infections is *Pseudomonas aeruginosa*. Burn injuries cause the skin barrier to be compromised, making it vulnerable to opportunistic infections. *P. aeruginosa* possesses various important virulence factors along with a high antibiotic resistance pattern that may contribute to its pathogenesis and limit acceptable treatment alternatives. **Objectives:** Investigate the distribution of (*toxA, algD, rh1I, pilA,* and *pilB*) genes in extensively drug-resistant (XDR) *P. aeruginosa* that are isolated from patients suffering from burn. **Materials and Methods:** A total of 60 burn swabs were taken from patients in the Specialized Burn Center in Al-Diwaniyah City, Iraq, from December 2021 to April 2022. *P. aeruginosa* isolates were identified by selective medium Chromagar™ *Pseudomonas* and API 20E system and confirmed by using the *16SrRNA* gene. Antibiotic susceptibility testing was performed for twelve agents of antibiotics in accordance with the recommendations of the Clinical and Laboratory Standards Institute (CLSI-2022). To detect the genes under study, the polymerase chain reaction technique was used. **Results:** A total of 16 isolates of *P. aeruginosa* from burn swabs were isolated. Antibiotic resistance patterns were found in all isolates; 10/16 (62.5%) isolates were extensively drug-resistant (XDR), whereas 6/16 (37.5%) isolates were multidrug-resistant. The results of the molecular study indicate that 10/10 (100%) of XDR *P. aeruginosa* isolates harbored the *toxA, algD,* and *rhlI* genes, whereas 9/10 (90%) and 8/10 (80%) of these isolates harbored *pilA* and *pilB* genes, respectively. **Conclusions:** XDR *P. aeruginosa* is one of the major active bacteria in burn patients. The high prevalence of virulence genes among XDR *P. aeruginosa* isolates plays an important role in *P. aeruginosa* pathogenesis in burn infection. As a result, new strategies such as vaccines, anti-virulence compounds, and antibody-drug conjugate are recommended measures to avoid the spread of drug-resistant *P. aeruginosa* in Al-Diwaniyah city hospitals.

Keywords: Antibiotic resistance patterns, burn, *Pseudomonas aeruginosa*, virulence genes

INTRODUCTION

Pseudomonas aeruginosa causes around 20% of hospital-acquired infections caused by Gram-negative bacteria, particularly in patients with burns. Burns are one of the most common types of skin or other tissue damage, that leads to the cause of mortality and morbidity worldwide.[1] *P. aeruginosa* may penetrate the bloodstream and spread to a variety of organ systems, resulting in grave and even fatal consequences including sepsis, organ failure, and shock.[2]

Antimicrobial resistance, according to the World Health Organization (WHO), is a significant global problem and is expected to develop into the greatest threat to public health by 2050, accounting for 10 million deaths per year.[3]

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Antibiotic-resistant bacteria, such as multidrug-resistant (MDR)/extensively drug-resistant (XDR) *P. aeruginosa*, have been identified as the primary reason for morbidity and mortality in burn patients.[4] Surprisingly, these strains

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have a high propensity to thrive in healthcare environments, notably in the care units of burns. As a result of broad-spectrum antibiotics being routinely utilized in these facilities, MDR and XDR *P. aeruginosa* are constantly emerging.[5] A mortality rate of 77% has been mentioned in burn patients caused by *P. aeruginosa* in the last 25 years.[1]

Pseudomonas aeruginosa pathogenicity is predicated on producing various cell- and biofilm-associated compounds such as pili, flagella, lipopolysaccharide, and alginate capsule. Also, it is able to secrete proteins, especially extracellular virulence factors (toxins and enzymes), that can play a probable role in the pathogenicity of *P. aeruginosa*.[6] These factors are involved in bacterial adhesion and colonization, host immunological suppression, and immune escape producing an antibiotic resistance barrier, and located on plasmids or chromosomes.[7] Exotoxin A is the major virulence factor produced by *P. aeruginosa* strains and is expressed by the gene *toxA*, it is a toxin that inhibits protein synthesis.[8] Alginate is encoded by the *algD* gene that plays a significant part in the survival of bacteria against the host's immune response and drugs that can disrupt the immune system against infection.[9] In *P. aeruginosa*, the *pilA* and *pilB* genes are essential to the production and assembly of type four pili, and mutation in these genes can impair the bacteria's capability to attach to surfaces, produce biofilm and cause infection.[10] The *rhII* gene is a component of the *rhl* quorum sensing (QS) system in *P. aeruginosa*, and it regulates the different virulence factors expression. This gene is responsible for producing the signaling molecule N-butyryl-homoserine lactone (C4-HSL), that is involved in the assortment of virulence gene expression.[11]

Considering the importance of the virulence genes of *P. aeruginosa* and also the antibiotic resistance that can be caused by this pathogen, this study aimed to investigate the distribution of (*toxA, algD, rh1I, pilA* and *pilB*) genes among XDR *P. aeruginosa* isolated from burn patients in Al-Diwaniyah city, Iraq.

Materials and Methods

Samples collection

A total of 60 burn swabs were collected from patients in the Specialized Burn Center in Al-Diwaniyah City, Iraq, using a sterile transport medium, from December 2021 to April 2022. The specimens were transported to the microbiological laboratory in Al-Hussein Hospital in Al-Diwaniyah City to conduct the necessary testing.

Identification of *P. aeruginosa*

The specimens were initially cultured on MacConkey agar and blood agar, and incubated at 37°C for 24 h. Then subcultured in Chromagar™ *Pseudomonas* as a selective medium. *P. aeruginosa* isolates were identified by conventional biochemical tests and confirmed by the API 20E system and *16SrRNA* gene.

Antibiotics susceptibility test

Susceptibility testing was performed on Müller-Hinton agar using the disk diffusion method according to CLSI recommendations. The antibiotics tested included piperacillin (100 μg), piperacillin-tazobactam (100/10 μg), cefepime (30 μg), ceftazidime (30 μg), imipenem (10 μg), meropenem (10 μg), aztreonam (30 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), gentamicin (10 μg), tobramycin (10 μg), and amikacin (30 μg). Inhibition zone diameters were evaluated according to CLSI guidelines.[12] The MDR phenotypes are defined as resistance to at least one antibiotic from three or more classes of antimicrobial agents. The isolates that were resistant to at least one antibiotic from each class of antibiotics used except for one or two classes were considered to be XDR.[13]

Molecular study

Genomic DNA extraction

DNA was extracted from *P. aeruginosa* isolates using (Easypure Genomic DNA Purification Kit, Trans China), a commercial extraction kit for Gram-negative bacteria extraction. A NanoDrop spectrophotometer was used to determine the purity of the isolated DNA at an absorbance ratio of 260/280 nm.

Amplification of 16SrRNA and virulence factor genes for P. aeruginosa

The primers sequences for *16SrRNA* and virulence genes are shown in Table 1. The amplifications were carried out in (25 μL) volume containing of PCR Master mix (12.5 μL), forward and reverse primers (1.5 μL), template DNA (3 μL) and nuclease-free water (6.5 μL). The PCR program was used to amplify all of the genomic regions mentioned in Table 2. Using agarose (1.5%) (Bioneer, Korea) containing ethidium bromide (0.5 μg/mL), the products of PCR were separated by agarose gel electrophoresis and observed using a UV transilluminator. The proper amplicon size was determined using a DNA ladder.

Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. The study protocol and subject information were reviewed and approved by a local ethics committee at Al-Diwaniyah Health Directorate, Iraq, according to document No. 227 on December 10, 2021.

Results

Out of 60 burn swabs, 38 (63%) gram-negative bacterial isolates were obtained. Only 16/38 (42.1%) of *P. aeruginosa* isolates were identified by conventional biochemical testing and diagnosis using Chromagar™ *Pseudomonas* selective medium [Figure 1] and the API 20E system and confirmed by *16SrRNA* gene.

Table 1: A list of the primers that were used to amplify genes

Primers		Sequence (5'-3')	Product size (bp)	Annealing temperature (ºC)	Reference
16 SrRNA	F	CGCCGTTAAACGATGTCGAC	510 bp	55	[14]
	R	CAGACTGCGATCCGGACTAC			
toxA	F	GGTAACCACGTCAGCCACAT	352 bp	55	[15]
	R	TGATGTCCAGGTCATGCTTC			
algD	F	CGTCTGCCGCGAGATCGGCT	313 bp	58	[16]
	R	GACCTCGACGGTCTTGCGGA			
rh1I	F	CTTGGTCATGATCGAATTGCTC	625 bp	50	[17]
	R	ACGGCTGACGACCTCACAC			
pilA	F	ACAGCATCCAACTGAGCG	1675 bp	59	[16]
	R	TTGACTTCCTCCAGGCTG			
pilB	F	TCGAACTGATGATCGTGG	408	54	[16]
	R	CTTTCGGAGTGAACATCG			

Table 2: PCR amplification program

Step	Temp.	Time	No. of cycle
Initial denaturation	94ºC	5 min	1
Denaturation	94ºC	1 min	32
Annealing	50-59ºC	1 min	
Extension	72ºC	1.5 min	
Final extension	72ºC	5 min	1
Hold temperature	4ºC	–	–

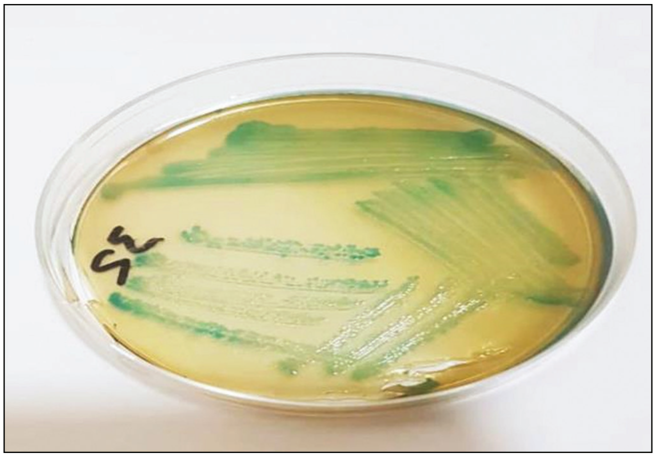

Figure 1: *P. aeruginosa* colonies on Chromagar™ *Pseudomonas*

Antibiotics susceptibility testing

To estimate the possible resistance of *P. aeruginosa* isolates for twelve agents of antibiotics [Figure 2]. All 16 isolates were tested for antibiotic susceptibility test according to (CLSI-2022) guidelines, and this test was accomplished by disc diffusion technique. The results showed that the tested isolates showed high degrees of resistance and patterns of susceptibility to these antibiotics. All isolates showed a complete resistance 16/16 (100%) for piperacillin, ceftazidime, cefepime, gentamicin, and tobramycin. Followed by 13/16 (81.3%) for ciprofloxacin, levofloxacin, aztreonam, and amikacin. 11/16 (68.7%) meropenem, 10/16 (62.5%) for Imipenem, and 8/16 (50%) for piperacillin–tazobactam. In addition, this study determined the prevalence of *P. aeruginosa* phenotype based on antibiotic resistance patterns [Figure 3]. The results demonstrated that non-susceptibility to five or six antibiotic classes was observed in 10/16 (62.5%) isolates, which was characterized as XDR. Moreover, non-susceptibility to four antibiotic classes was observed in 6/16 (37.5%) isolates, which were characterized as MDR *P. aeruginosa.*

Molecular study

Detection of 16SrRNA species-specific primers for P. aeruginosa

All sixteen *P. aeruginosa* DNA samples were molecularly identified using the PCR. The results presented in Figure 4 showed that all 16/16 (100%) isolates contained *16S rRNA* gene with molecular weight (510 bp).

Detection of virulence factor genes

Molecular analysis was used to screen five virulence genes (*toxA, algD, rh1I, pilA,* and *pilB*) in ten *P. aeruginosa* burn isolates that were XDR.

The result showed that all 10 (100%) *P. aeruginosa* isolates gave positive and specific bands of *toxA, algD,* and *rh1I* genes [Figures 5–7], followed by 9 (90%) and 8 (80%) from these isolates were carried *pilA* and *pilB* genes, respectively [Figures 8 and 9].

DISCUSSION

Pseudomonas aeruginosa is a frequent source of burns inflammation and can be found in soil, water, small

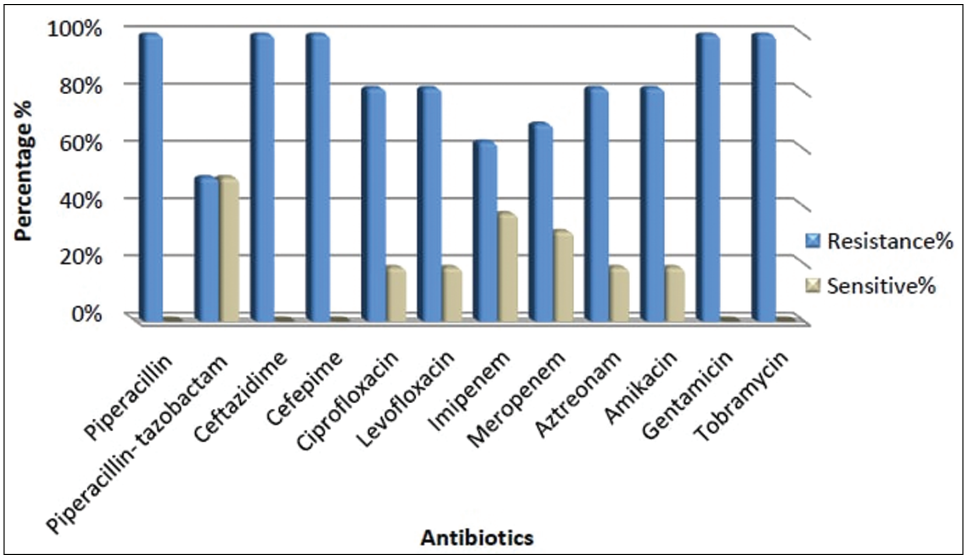

Figure 2: Patterns of antibiotic susceptibility in *P. aeruginosa* burn isolates

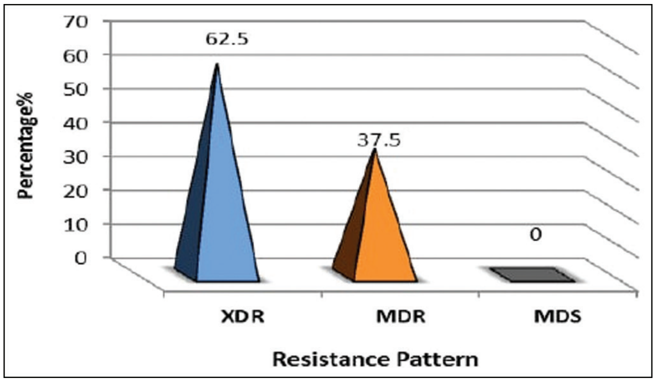

Figure 3: Prevalence of resistance patterns of *P. aeruginosa* burn isolates

amounts in the intestines, and the moist environments in hospitals because it thrives in these conditions. They are the main contributors to burn infections.[18,19]

In this study, 16 (42.1%) of *P. aeruginosa* isolates were obtained from burn swabs. The immunosuppression cases caused by burn trauma, as well as the wound local milieu, are both favorable for pathogen colonization and multiplication. Among the microorganisms in burns, *P. aeruginosa* is the most common and often becomes the main pathogen of established infection.[20] On the other hand, sepsis is a major cause of death in burn patients. *P. aeruginosa* impacts the blood metabolome through the spread from infected burns and subsequent sepsis, as a result of alterations to particular metabolites that could act as biomarkers to aid in early detection of sepsis that is caused by *P. aeruginosa*.[21]

The antibiotic susceptibility test results were as follows: a complete resistance of 16 (100%) for piperacillin, ceftazidime, cefepime, gentamicin, and tobramycin, respectively. Followed by 13 (81.3%) for ciprofloxacin, levofloxacin, aztreonam, and amikacin, respectively. 11 (68.7%) meropenem, 10 (62.5%) for Imipenem, and 8 (50%) for piperacillin–tazobactam. The β-lactams are essential in the treatment of infections caused by *P. aeruginosa*. Despite that, a considerable number of *P. aeruginosa* are resistant to these antibiotics, complicating infection therapy and contributing to bad patient outcomes, these resistance can include alterations to penicillin-binding protein, which is required for decreased uptake or increased efflux of antibiotics, degeneration of β-lactams via increasing expression or changed substrate specificity of β-lactamases (AmpC) or horizontal gene transfer that causes the acquisition of β-lactamases, as well as alterations in metabolism and biofilm production. The reason for aminoglycoside resistance can be taken by aminoglycoside-modifying enzymes, which impact the structural integrity of these antibiotics to varying degrees by catalytic alter, or by RNA methyltransferases that can change the target site of *16S rRNA*. Target site changes in *parC* and *gyrA*, which are components of topoisomerase IV and DNA gyrase, respectively, can lead to fluoroquinolone resistance.[22]

A local study in Duhok recorded resistant percentages (100%) to piperacillin, ceftazidime, and cefepime, respectively, followed by 79.2% and 64.6% to meropenem and tobramycin, respectively.[23] Also, a study in Baghdad recorded resistant percentages 85.5%, 73.9%, 76.8%, 71%, 71%, and 63.8% for piperacillin, gentamicin, cefepime, imipenem, tobramycin, and meropenem, respectively.[24] While the other study done by Al-Wahid[25] in Al-Diwaniyah recorded resistant percentages 39%, 35%, 31%, 29%, 23%, 21%, 18%, 16% and 13% for cefepime, ceftazidime, piperacillin, aztreonam, gentamicin, ciprofloxacin, imipenem, meropenem, and amikacin, respectively. The variance in resistance levels between studies can be linked to variations in geographical distribution, sample sizes used in studies, and antibacterial laws in various places of the world.

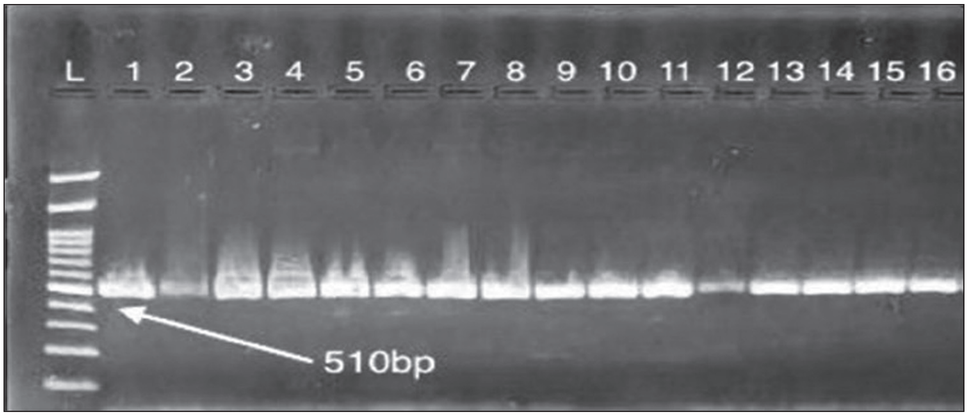

Figure 4: Agarose gel electrophoresis for *P. aeruginosa 16S rRNA* gene with product size (510 bp) in PCR at 75 V for 90 min in 1.5% agarose. Lane (L): 100 bp DNA ladder, lanes (1–16) positive results for amplification

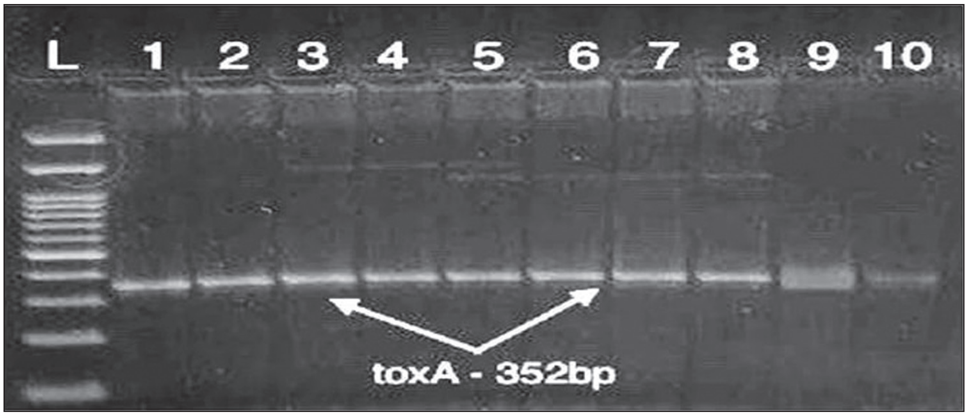

Figure 5: Agarose gel electrophoresis of PCR product of *toxA* gene at (352 bp) of XDR *P. aeruginosa* isolates. L: 100 bp DNA ladder, lanes (1–10) positive results for amplification, the electrophoresis was at 70 V for 80 min

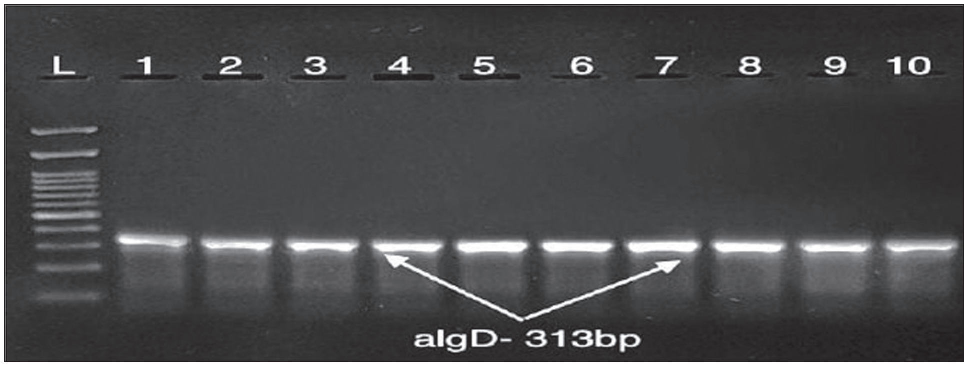

Figure 6: Agarose gel electrophoresis of PCR product of *algD* gene at (313 bp) of XDR *P. aeruginosa* isolates. L: 100 bp DNA ladder, lanes (1–10) positive results for amplification, the electrophoresis was at 70 V for 80 min

The frequency of resistance against antibiotic classes was investigated in this study, which revealed high resistance percentages among antimicrobial agents, with 62.5%, 37.5%, and 0% of *P. aeruginosa* isolates displaying XDR, MDR, and MDS resistance phenotypes, respectively. This high resistance pattern is troubling.

The MDR/XDR *P. aeruginosa*, which carries several virulence genes, has emerged as a global public health threat. According to the results of the present study, 100% of ten XDR *P. aeruginosa* isolates were *toxA*, *algD,* and *rhII* genes positive, respectively whereas 90% and 80% of isolates were *pilA* and *pilB* genes positive, respectively. Exotoxin A is a pathogenic *P. aeruginosa* extracellular product expressed by the *toxA* gene on their chromosome. It works by inhibiting protein biosynthesis in the host cell, similar to how diphtheria toxin works.[26] Ghazaei[27] mentioned that exotoxin A, which is a NAD+-diphthamide

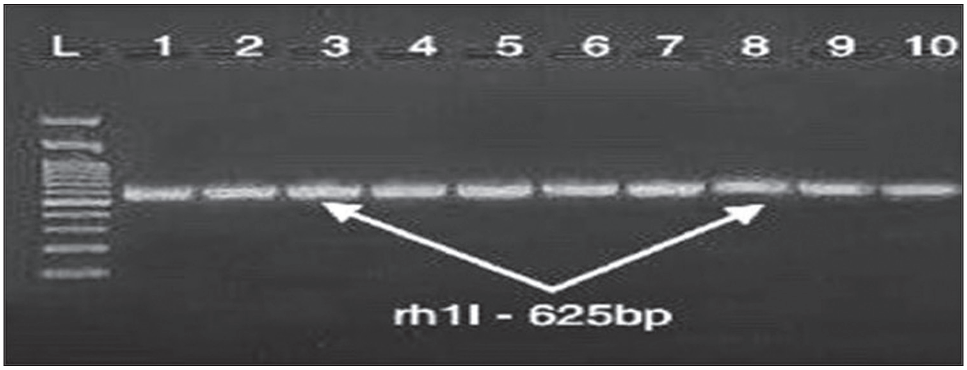

Figure 7: Agarose gel electrophoresis of PCR product of *rhlI* gene at (625 bp) of XDR *P. aeruginosa* isolates. L: 100 bp DNA ladder, lanes (1–10) positive results for amplification, the electrophoresis was at 70 V for 80 min

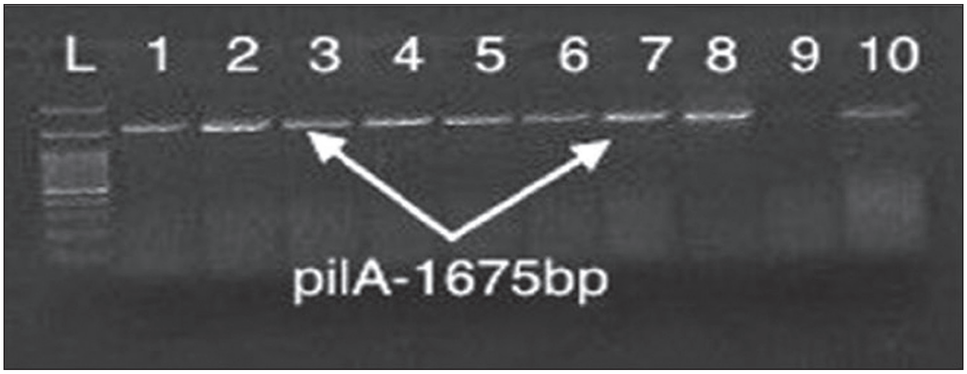

Figure 8: Agarose gel electrophoresis of PCR product of *pilA* gene at (1675 bp) of XDR *P. aeruginosa* isolates. L: 100 bp DNA ladder, lanes (1–8 and 10) had positive results, while lane (9) negative result for amplification, the electrophoresis was at 70 V for 80 min

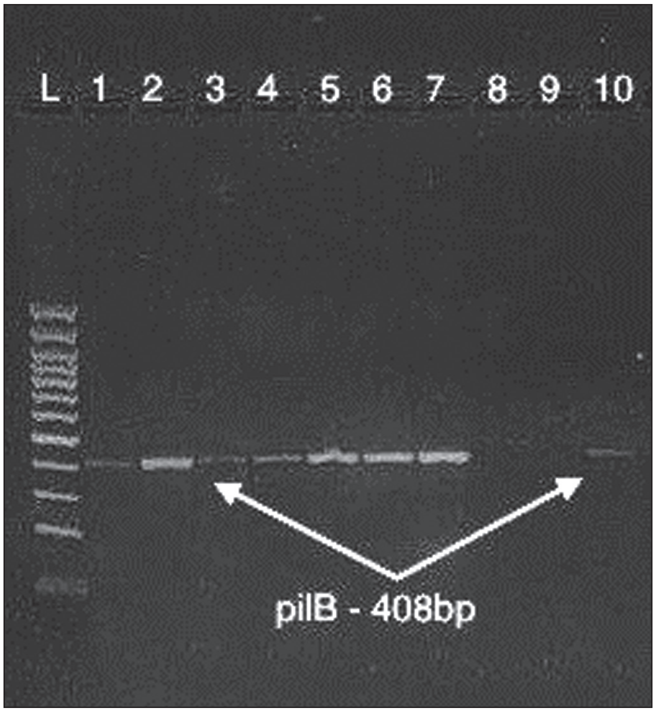

Figure 9: Agarose gel electrophoresis of PCR product of *pilB* gene at (408 bp) of XDR *P. aeruginosa* isolates. L: 100 bp DNA ladder, lanes (1–7 and 10) had positive results, while lanes (8, 9) negative result for amplification, the electrophoresis was at 70 V for 80 min

ADP-ribosyl transferase, is one of the most effective virulence factors in *P. aeruginosa*. It catalyzes the transport of ADP-ribose moiety from NAD+ to the diphthamide remains on the eukaryotic elongation factor-2 (eEF-2) to impair protein synthesis and give rise to the death of host cells. (100%) of *P. aeruginosa* strains linked to burn infection were reported to have this gene distributed among them, according to.[28] In other studies, conducted by,[6,29] the PCR findings revealed 100% and 96.3% of the isolates carried the *tox*A gene, respectively. In addition, only 60% of isolates have the *tox*A gene represented by,[30] whereas[31] recorded 23% for this gene, respectively.

The *algD* gene is involved in the biosynthesis of alginate, an exopolysaccharide that forms the structural component of *P. aeruginosa* biofilms. Alginate protects bacteria from antibiotics and host immune responses. Mutations in *algD* result in defective alginate production and reduced biofilm formation, leading to attenuated virulence.[32] Alabdali and Alkaim[33] and Al-Shimmary[34] found that the prevalence rate of *algD* in *P. aeruginosa* burn isolates was 100% and 93.5%, respectively, whereas[35] have recorded a prevalence of 39%. It is likely that the observed discrepancies in this gene's prevalence are due to the fact that the dominant clones in each location differ.

Virulence factors and biofilm formation are operated by QS systems in *P.aeruginosa*. The QS has been shown to be implicated bacterial species. *Las* and *rhl* are two hierarchical QS systems found in *P. aeruginosa*.[36] The *rhl* system regulates several virulence factors like biofilm development and alkaline protease via increasing the biosynthesis of polysaccharide, pyocyanin, elastase, and rhamnolipid.[37] In the local study done by[38] the *rh1I* gene was detected in 56 (93.33%) clinical *P. aeroginosa* isolates, whereas[39] recorded 43% for this gene in clinical *P. aeruginosa* isolates.

The *pilA* and *pilB* genes encode components of the type IV pili, which are crucial for bacterial adhesion, twitching motility, and biofilm formation. These pili facilitate the primary attachment of bacteria to the host tissues and enable the formation of microcolonies.[40] Mutations in both genes have been demonstrated to impact *P. aeruginosa's* capacity to produce biofilm, attach to surfaces, and cause infection. Many investigations discovered that a mutant *P. aeruginosa* strain with a deletion in the *pilA* gene was unequal to produce biofilm on abiotic surfaces and had decreased virulence in infection mice models.[10,41]

CONCLUSIONS

The current investigation found that *P. aeruginosa* isolates revealed high resistance toward antibiotics under study. Also indicated that XDR *P. aeruginosa* is one of the major active bacteria in burn victims. This study revealed the virulence gene prevalence (*toxA, algD, rh1I, pilA,* and *pilB*) among XDR *P. aeruginosa* isolates. Furthermore, the high prevalence of these genes is significant in *P. aeruginosa* pathogenesis in burn infections. These findings could result in the emergence of resistant *P. aeruginosa* isolates that are capable of infecting patients as well as healthy persons in hospitals. As a result, the optimization of antibiotic use and infection management by using new strategies such as vaccines are recommended measures to avoid the spread of drug-resistant *P. aeruginosa* in Al-Diwaniyah City hospitals.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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