RESEARCH ARTICLE

Molecular and Virulence Analysis of Pseudomonas aeruginosa Isolated From Burn Infections Recovered From Duhok and Erbil Hospitals, Iraq

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ABSTRACT

In this study, 225 isolates of *Pseudomonas aeruginosa* were recovered from burn wounds in major hospitals in Duhok and Erbil, Iraq, between April 2015 and September 2015. A total of 136 of these isolates were from men, comprising 60.4% of the total, whereas 89 (39.6%) were recovered from women. One hundred of these isolates were selected (50 from each province of Erbil and Duhok) and subjected to 16 different antibiotics using the disc diffusion method. The isolates showed a high level of resistance to most of the tested antibiotics, with 90% of the isolates being multidrug resistant. Imipenem was considered as the most effective antibiotic against these isolates with a resistant rate of 47%. The genome of all of these isolates were successfully amplified and produced a single band for the *16S rDNA* locus with a molecular weight of about 956 base pairs, which was used to confirm, at the molecular level, that all these isolates were indeed *P. aeruginosa.* The results of the detection of five virulence-related genes including *opr1, toxA, exoS, lasB,* and *nan1* revealed that 10 of these isolates, accounting for 10%, lacked any of the tested virulence markers. The *opr1* gene, as a marker for the presence of a pathogenicity island, was the most dominant marker among all the virulence markers and was detected in 90 isolates (90%), followed by the *toxA* and *exoS* genes, which were both observed in 86 (86%) isolates, whereas the *lasB* gene was found in 82 (82%) isolates and the *nan1* gene in 35 (35%) of the isolates, respectively.

Keywords: Burn infection, P. Aeruginosa, PCR, 16s rDNA, Virulence genes

1. INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that infects organisms and causes nosocomial infections (Vincent et al., 2004). It is one of the major causes of chronic lung infections in patients with cystic fibrosis (CF) and a major cause of hospital-acquired infections (Stover et al., 2000).

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P. aeruginosa is well suited to survive in a wide variety of environments including water, soil, and animals, and it is prevalent in common, everyday surroundings (David et al., 2007). P. aeruginosa infections may occur in patients with cancer, patients suffering from urinary tract infections, and patients suffering from burn wounds (Bodey et al., 1983). Infections caused by *P. aeruginosa* are often difficult to treat because of the prominent resistance exhibited by the pathogen to antimicrobial agents (Hancock, 1998). In the context of a breakdown in host defenses, it is capable of infecting a plethora of tissue types, causing both acute and chronic infections. Burn victims as well as immunocompromised, mechanically ventilated, and patients with CF are particularly susceptible to *P. aeruginosa* infections (Sadikot et al.,

2005). *P. aeruginosa* exhibits a variety of virulence factors to overcome the host defenses and establish an infection. These factors include the production of hemolysin, pyocyanin, gelatinase, and the formation of biofilms, which act by increasing tissue damage and helping the bacteria to evade the immune system and to avoid the action of antibiotics (Cevahir et al., 2008). The pathogenesis of the infections is multifactorial, as suggested by the number and broad range of virulence determinants expressed by the bacterium (Todar, 2009). *P. aeruginosa* is notorious for its multiple virulence factors such as adhesins, biofilm formation, elastase production, surface hemagglutinin, motility, synthesis and production of pyocyanin, rhamnolipid, type III secretion system, colonization pili, lipopolysaccharide, flagella, alkaline protease, siderophore uptake systems, and extracellular protein toxins (e.g., exoenzyme S and exotoxin A) (Gallagher and Manoil, 2001).

2. MATERIALS AND METHODS

A total of 225 clinical isolates of *P. aeruginosa* from infected burn wounds were collected from different patients attending major hospitals in Duhok and Erbil, Iraq. The patient population comprised both sexes, different ages, and different percentages of burn coverage during the period from April 2015 to September 2015. On the basis of the clinical judgment of the infection, swabs of pus from an infected burn wound were collected at the same time the dressings were changed. All of the clinical isolates, suspected to be *P. aeruginosa*, which were collected from the infected burn wouds were recultured onto various media including MacConkey agar, cetrimide agar, blood agar, and nutrient agar by the streak plate method and incubated at 37˚C for 24 hours (Cheesbrough, 2006). The isolates were subjected to antibiotic sensitivity testing by the disc diffusion method on Mueller-Hinton agar according to the National Committee for Clinical Laboratory Standards and the Manual of Antimicrobial Susceptibility Testing (Cheesbrough, 2006; CLSI, 2007). Genomic DNA was extracted from 100 *P. aeruginosa* strains using a high yield DNA purification kit according to the manufacturer instructions (Bioneer, Daejeon, Republic of Korea).

The results of the DNA extraction were visualized using ultraviolet light after being electrophoresed on a 1% agarose gel (Maniatis et al., 1982). Table 1 shows the sequences of the primers and the amplification band sizes for the *16S rDNA*, *nan1*, *exoS*, *lasB*, *toxA*, and *opr1* genes, which were used for polymerase chain reaction (PCR) amplification by adding 12.5 µl of master mix (GeneDirex, USA), 1 µl of each primer including the forward and reverse primers (10 pmol/µl), 4 µl of genomic DNA (25–50 ng/µl), and 6.5 µl of sterile, deionized distilled water to each reaction. All of the prepared reaction tubes were placed in the thermal cycler to carry out the amplification.

The amplification conditions are illustrated in Table 2. The presence of the PCR product was confirmed electrophoretically using a 1.5% (w/v) agarose gel in tris-borate-ethelenediaminetetraacetic acid buffer. A molecular marker of 1,500–100 base pairs (bp) was used to determine the molecular weights of the PCR products (Maniatis et al., 1982).

Table 1: Molecular weights of the genes and sequences of the primers used

Gene	Forward and Reverse primer	Size product (bp)	Refrences
16S rDNA	F 5′ GGGGGATCTTCGGACCTCA 3′	956	Spilker et al., 2004
	R 5′ TCCTTAGAGTGCCCACCCG 3′		
Nan1	F 5′ AGGATGAATACTTATTTTGAT 3′	1316	Stover et al., 2000
	R 5′ TCACTAAATCCATCTCTGACCCGA 3′		
ExoS	F 5′CTTGAAGGGACTCGACAAGG 3′	504	Stover et al., 2000
	R 5′TTCAGGTCCGCGTAGTGAAT3′		
LasB	F 5′GGAATGAACGAAGCGTTCTC3′	300	Stover et al., 2000
	R 5′GGTCCAGTAGTAGCGGTTGG3′		
ToxA	F 5′GACAACGCCCTCAGCATCACCAGC3′	396	Rawya et al., 2008
	R 5′CGCTGGCCCATTCGCTCCAGCGCT3′		

Opr1	F 5´ATGAACAACGTTCTGAAATTCTCTT3´ R 5´CTTGCGGCTGGCTTTTTCCAG3´	249	De Vos et al., 1993

Table 2: PCR amplification conditions for the *16S rDNA, Nan1, ExoS, LasB, ToxA*, and*Opr1* genes of *Pseudomonas* spp.

Gene	Initial denaturation	Denaturation	Annealing	Extension	Final extension
16S rDNA	95°C; 2min;	94°C; 20 s	54°C; 20 s;	72˚C; 40 s	72°C; 5 min;
	1 cycle		25 cycles		1 cycle
Nan1	94°C; 5 min	94°C; 20 s	54°C; 1 min	72˚C; 1min	72°C; 90 s
	1 cycle		36 cycles		1 cycle
ExoS	95°C; 2 min	94°C; 30 s	60°C; 1 min	72˚C; 1 min	72°C; 5 min
	1 cycle		35 cycle		1 cycle
LasB	94°C; 3 min	94°C; 30 s	60°C; 1 min	72˚C; 90 s	72°C; 5 min;
	1 cycle		30 cycle		1 cycle
ToxA	94°C; 2 min	94°C; 2 min	68°C; 1 min	72˚C; 1 min	72°C; 7min
	1 cycle		30 cycle		1 cycle
Opr1	95°C; 2 min	94°C 40 s	57°C; 50 s	72˚C; 20 s	72°C; 5 min
	1 cycle		25 cycles		1 cycle

3. RESULTS

The purified isolates that grew on MacConkey and cetrimide agar confirmed that the isolates were indeed *P. aeruginosa* and that the tests were not contaminated during transport from the respective hospitals in Erbil and Duhok, Iraq. These isolates were found to be negative for lactose fermentation and formed pale yellow colonies on MacConkey agar and produced β-hemolytic colonies on blood agar. The colonies were surrounded by a bluish-green coloration on nutrient agar because of the production of soluble pyocyanin and pyoverdine, which is a water-soluble, yellow-green pigment. The pigments produced by the colonies on the selective cetrimide agar were more obvious (Prescott et al., 1993). The identity of the *P. aeruginosa* isolates from infected burn wounds were confirmed by biochemical tests such as the oxidase test, citrate utilization test, and the ability to grow at a temperature of 42˚C (Cunliffe et al., 1995).

Table 3: Samples of *P. aeruginosa* collected from 2 areas in Iraq and their prevalence among male and females patients

Provinces	Number of patients	Male, n (%)	Female, n (%)
Erbil	125	72 (57.6)	53 (42.4)
Duhok	100	64 (64)	36 (36)
Total	225	136 (60.4)	89 (39.6)

The patient demographics for the 225 patients from which the *P. aeruginosa* isolates were obtained are depicted in Table 4 and include the cause of the burn wounds, age, sex, and the total burn surface area (TBSA). Burns from a flame was the most common cause of the burn wounds, representing 125 (55.5%) of the patients, whereas scalding was the second most common cause of burn wounds, representing 85 (37.7%) of the patients. The remaining 15 patients (6.6 %) had chemical (acid) burns. Forty two out of the 125 patients (33.6%) whose burn wounds were caused by fire were aged 12 years and below, while the remaining 83 (66.4%) patients were older than 12 years. Of those patients with burn wounds caused by fire, 88 (70.4%) were male and 37 (29.6%) were female. The TBSA for the patients with fire burns was less than 15% for 29 (23.2%) of the patients, whereas the remaining 96 (76.8%) patients had a TBSA of >15%. A total of 33 of the 85 patients (38.8%) who obtained burn wounds from scalding were younger than 12 years, whereas the remaining 52 (61.1%) patients were older than 12 years. Of the patients with burn wounds from scalding, 43 (50.5%) were male and the remaining 42 (49.4%) patients were female. The TBSA for the patients with burn wounds from scalding were <15% for 13 (15.2%) of the patients and >15% for the remaining 72 (84.7%) patients. Of the 15 patients who had burn wounds from acid, 4 (26.6 %) were younger than 12 years, whereas the remaining 11 (73.3%) patients were older than 12 years. The patients with acid burns were mostly male, representing 12 (80%) of the patients, whereas the remaining 3 (20%) patients were female. The TBSA was <15% for 5 (33.3%) of the 15 acid burn patients, whereas the remaining 10 (66.6%) patients had a TBSA of >15%.

Table 4: The cause of the burn wounds, age, sex, and the tbsa for the 225 patients from which the *P. aeruginosa* isolates were obtained

Cause of burn	Patients, n (%)	Age		Sex		TBSA	
		<12 years (n=79)	>12 years (n=146)	Male (n=143)	Female (n=82)	<15% (n=47)	>15 % (n=178)
Flame	125 (55.5)	42 (33.6)	83 (66.4)	88 (70.4)	37 (29.6)	29 (23.2)	96 (76.8)
Scald	85 (37.7)	33 (38.8)	52 (61.1)	43 (50.5)	42 (49.4)	13 (15.2)	72 (84.7)
Acid	15 (6.6)	4 (26.6)	11 (73.3)	12 (80)	3 (20)	5 (33.3)	10 (66.6)

The results of the antimicrobial sensitivity tests for the *P. aeruginosa* isolates, representing 50 of the isolates from each of the Duhok and Erbil territories, are shown in Table 5. It is clear that none of the isolates were sensitive to all the antimicrobials. Imipenem, an antimicrobial from the carbapenem class of antibiotics, was found to be the most powerful among all other antimicrobials with a combined resistance rate of 47% across the 2 districts.

Table 5: Resistance distribution against the tested antibiotic for the *P. aeruginosa* isolates from Duhok and Erbil

Antibiotics	Class	Duhok Resistance, n (%)	Erbil Resistance, n (%)	Total Resistance, n (%)

Amikacin	Aminoglycoside	46 (92)	45 (90)	91 (91)
Tobramycin	Aminoglycoside	48 (96)	46 (92)	94 (94)
Gentamicin	Aminoglycoside	46 (92)	47 (94)	93 (93)
Ticarcillin-clavulanic acid	Beta-lactam	35 (70)	36 (72)	71 (71)
Ampcillin-sulbactam	Beta-lactam	40 (80)	43 (86)	83 (83)
Ampcillin	Penicillin	43 (86)	42(84)	85 (85)
Piperacillin	Penicillin	44(88)	41 (82)	85(85)
Meropenem	Carbapenem	47 (94)	48 (96)	95 (95)
Imipenem	Carbapenem	25 (50)	22(44)	47 (47)
Cefepime	Cephalosporin	49 (98)	44 (88)	93 (93)
Ceftazidime	Cephalosporin	45(90)	46 (92)	91 (91)
Ceftriaxone	Cephalosporin	45 (90)	45 (90)	90 (90)
Cefuroxime	Cephalosporin	46 (92)	44 (88)	90(90)
Ciprofloxacin	Fluoroquinolone	27 (54)	29 (58)	56 (56)
Aztreonam	Monobactam	43 (86)	47(94)	90 (90)
Trimethoprim-sulfamethoxazole	Folate pathway inhibitor	23 (46)	31 (62)	54 (54)

Meropenem may be considered as the antibiotic with the highest resistance rate among the *P. aeruginosa* isolates, with 95 (95%) of the isolates across both territories displaying resistance. The *P. aeruginosa* isolates showed a high level resistant to most of the tested antimicrobials with the resistances rates for tobramycin, gentamicin, amikacin, and aztreonam being 94%, 93%, 91%, and 90%, respectively. Resistance against ampicillin and piperacillin, which are usually considered as first-line treatments for *P. aeruginosa* infections of burn wounds, seemed to be widespread with a total resistance rate for each of the antibiotics of 85% across the 2 territories. The resistance rates for the drug combinations ticarcillin-clavulanic acid and ampicillin-sulbactam was found to be in the range of 71% and 83%, respectively. The trimethoprim-sulfamethoxazole combination and ciprofloxacin seemed to have a relatively low resistance rate with 54% and 56% of the strains across both territories displaying resistance, respectively. There was a high level of resistance against the fourth generation cephalosporins with cefepime, ceftazidime, ceftriaxone, and cefuroxime having resistance rates of 93%, 91%, 90%, and 90%, respectively.

In the PCR tests, the *16S rDNA* gene was successfully amplified for all of the 100 *P. aeruginosa* isolates, generating a single band at a molecular weight of around 956 bp, and providing further evidence that all of the strains that were isolated were indeed *P. aeruginosa.* These results are depicted in Fig. 1.

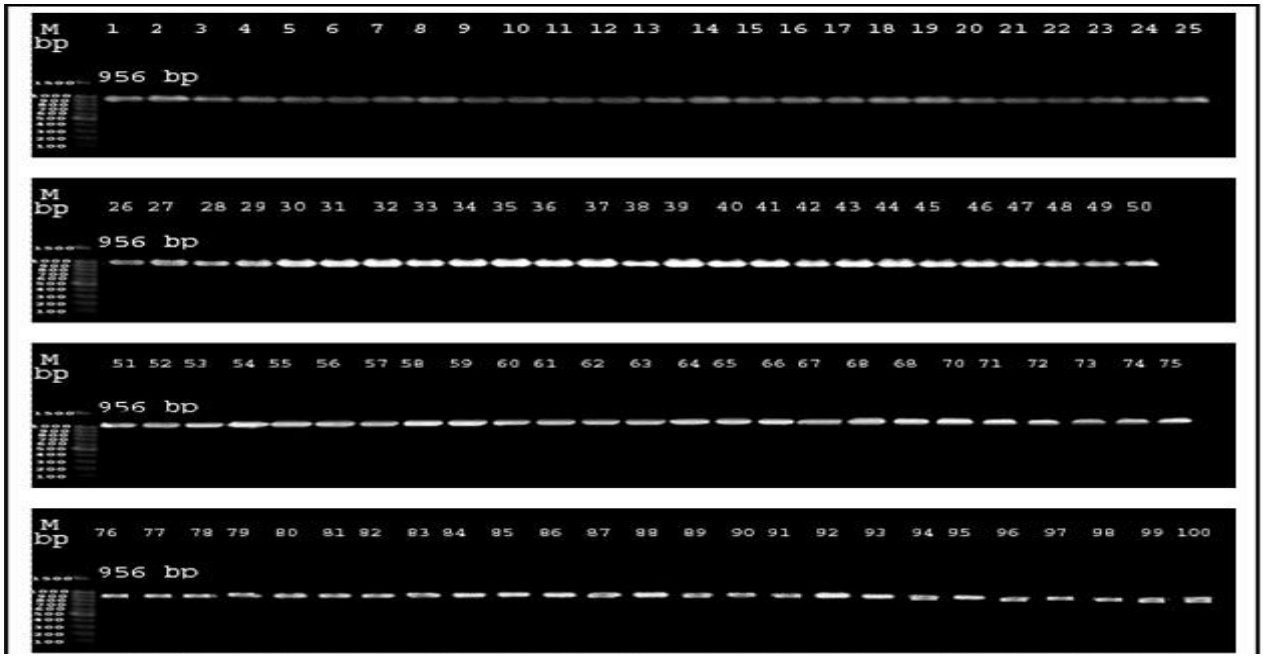

Figure 1. The PCR products, observed as bands, that correspond to amplification of the *16S rDNA* gene of *P. aeruginosa* after electrophoresis on 1.5% agarose gels and run at 5 V/cm for 1.30 hour. Line M contained the DNA marker (1,500–100 bp).

The prevalence rates of the virulence-related genes *opr1*, *toxA*, *exoS*, *lasB*, and *nan1* in the *P. aeruginosa* isolates were investigated using PCR amplification and the results of these amplifications are displayed in Figs. 2, 3, 4, 5, and 6 and Table 6.

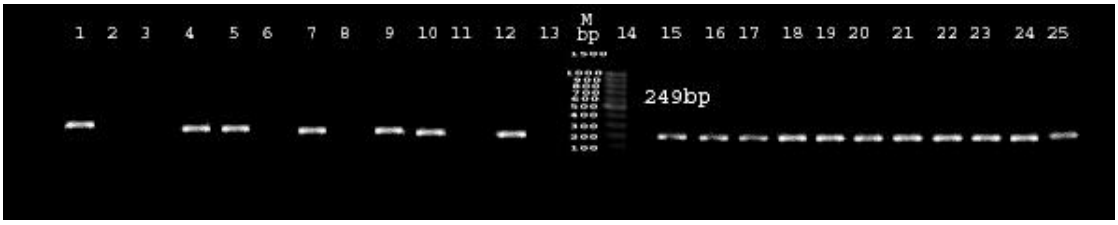

Figure 2. PCR amplification of the *opr1* gene with a molecular weight of 249 bp following electrophoresis on a 1% agarose gel that was run at 5 V/cm for 1.30 hours. Line M contained the DNA molecular weight marker (1,500–100bp).

Figure 3. PCR amplification of the *toxA* gene with a molecular weight of 396 bp following electrophoresis on a 1% agarose gel that was run at 5 V/cm for 1.30 hours. Line M contained the DNA molecular weight marker (1,500–100bp).

Figure 4. PCR amplification of the exoS gene with a molecular weight of 504 bp folloinwg electrophoresis on a 1.5% agarose gel that was run at 5 V/cm for 2 hours. Line M contained the DNA molecular weight marker (1,500–100bp)

Figure 5. PCR amplification of the *lasB* gene with a molecular weight of 300 bp following electrophoresis on a 1% agarose gel that was run at 5 V/cm for 1.30 hours. Line M contained the DNA molecular weight marker (1,500–100bp).

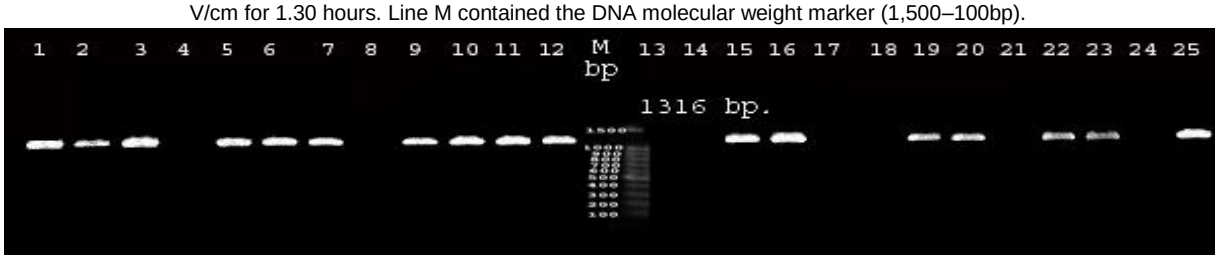

Figure 6. PCR amplification of the *nan1* gene with a molecular weight of 1,316 bp following electrophoresis on a 1% agarose gel that was run at 5 V/cm for 1.30 hours. Line M contained the DNA molecular weight marker (1,500–100bp)

Table 6: Prevalence of the virulence markers among the *P. aeruginosa* isolates collected from the Erbil and Duhok provinces

Province	Virulence markers				
	***oprI*, n (%)**	***toxA*, n (%)**	***exoS*, n (%)**	***lasB*, n (%)**	***nan1*, n (%)**
Erbil	45(90)	44(88)	43(86)	42(84)	19(38)
Duhok	45(90)	42(84)	43(86)	40(80)	16(32)
Total	90(90)	86(86)	86(86)	82(82)	35(35)

4. DISCUSSION

From the results presented in Table 3 it can be seen that of the 225 isolates of *P. aeruginosa* that were collected from various hospital in 2 areas (Erbil and Duhok) in Iraq, 143 were isolated from men, accounting for 63.5% of the isolates, whereas 82 strains were isolated from women, accounting for 36.4% of the isolates. On the basis of the results obtained to create a better understanding of the patient demographics for comparison to other studies, presented in Table 4, it was observed that the most common cause of burn wounds in these regions was fire, with 25 (80.6 %) of the patients being burned with fire. Scalding and acid burns were the second and third most common causes of burns, respectively. The most common patient demographics associated with an increased rate of

P. aeruginosa infection, irrespective of the cause of the burns, were male patients older than 12 years of age and a TBSA >15% (Naqvi et al., 2005). The TBSA was found to be the greatest risk factor for nosocomial infections (Oralancul et al., 2002). *P. aeruginosa* remains the most common pathogen associated with burn wound infections (Lari and Bahrami, 1998). It survives well in the clinic environment. Once *P. aeruginosa* colonization has been established, it can survive for months inside a unit and pose a threat as a multidrug resistant pathogen that can cause nosocomial infections in the patients being treated there. The hands of staff members can be contaminated with the bacteria, which can then be spread readily among patients (Edwards, 2003). In previous studies, the effectivity of imipenem against *P. aeruginosa* was moderately greater, i.e., 86%, 78%, 88,%, and 91.6%, respectively (Neely and Holder, 1999). The resistance of the *P. aeruginosa* isolates against imipenem was much higher in our study (48 %) than that reported in a previous study conducted by Song et al. (2001) in Korea.

The rates of resistance against these drugs should be considered in the treatment of serious bacterial infections caused by β-lactam resistant bacteria (Paterson, 2006). The development of carbapenem resistance owing to the generation of carpabenemase compounds in Gram negative organisms is increasing universally. The discovery of carbapenem resistance in the hospital environment poses a great risk for infection that needs to be controlled (Hodiwala et al., 2013). Carbepenems are valuable in the treatment of a few cases of multidrug resistant strains of *P. aeruginosa* (Douglas, 2001).

Comparative studies about the use of antimicrobial agents in the treatment of patients with multidrug resistant infections are limited, but a few of these agents remain valuable in the treatment of specific patients (Nicolle, 2005). In another study comparing the effectivity of a range of antimicrobial agents in the treatment of *P. aeruginosa* infections, 100% of the tested strains were resistant to amikacin, 95% were resistant to gentamicin, 94% were resistant to aztreonam, and 91% were resistant to tobramycin (Naqvi et al., 2005). In a comparative study conducted in Pakistan in which the effectivity of ampicillin and piperacillin was also determined in the treatment against *P. aeruginosa*, it was found that 87% of the tested strains were resistant to ampicillin and piperacillin (Naqvi et al., 2005). The broad utilization of the fourth generation of cephalosporins is the driving force behind the rise in the extended-spectrum β lactamase (ESBL)-generating organisms and has been the focus of numerous studies (Paterson, 2006). It has been found that the genes that encode the ESBLs are habitually found on the same plasmids as the genes that encode resistance to the aminoglycosides and trimethoprim-sulfamethoxazole (Yasufuku et al., 2011).

This implies that ESBL producing bacteria are commonly multidrug resistant, which poses a specific challenge in the treatment of nosocomial infections. Unsuccessful antimicrobial treatment of nosocomial- or community-acquired infections has been established to contribute to the mortality rates in intensive care units. Unsuccessful antimicrobial treatment of infections was the most important determinant of the mortality rates in hospitals (Paterson, 2006). Numerous previous studies have reported the successful amplification of the *16S rDNA* gene as shown in Fig. 1, and this has been deemed a prerequisite for the molecular identification of *P. aeruginosa* (Theodore et al., 2004). The results obtained corresponded with those of a study conducted in the Baghdad Territory in which the same primers for the *16S rDNA* gene were used for the identification of *P. aeruginosa* and in which the same molecular weight band was obtained. The results displayed in Figs. 2 to 6 appear to correlate with a number of related studies, for example, Khattab et al. (2015) found that 100% of their *P. aeruginosa* isolates possessed the *opr1* gene, whereas the *nan1* gene had the lowest prevalence.

In another study conducted in Poland by Wolska and Szweda (2009), it appeared that the most common virulence genes among *P. aeruginosa* isolates obtained from burn wound infections were *lasB*, *toxA*, and *exoS*, with prevalence rates of 96.8%, 88.7%, and 75.8%, respectively. Fazeli and Momtaz (2012) also determined the prevalence of the virulence factors of *P. aeruginosa* isolates from burn wound infections and found that the prevalence rates for *exoS* and *toxA* were 67.6% and 35.2%, respectively. The differences between the prevalence rates of *P. aeruginosa* virulence genes based on the geographic location were investigated in studies conducted by Rasol (2013) in the Duhok territory and by Karimian et al. (2012) in Iran who concluded that the climate of each location, their traditions, their foods, levels of open well-being, and hospital cleanliness may all contribute to the differences in the prevalence rates of

virulence genes among the *P. aeruginosa* strains from various districts.

5. CONCLUSION

It can be concluded that molecular methods are a fast and effective way of confirming the identity of *P. aeruginosa* isolates. It is important to determine if multidrug resistant isolates of *P. aeruginosa* are present and if they pose a risk of spreading widely and increasing their resistance rates over time.

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