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Prevalence of *Pseudomonas aeruginosa* isolates with molecular identification of some antibiotic resistant genes in Hospitals of Duhok City/ Iraq

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ABSTRACT: *Pseudomonas aeruginosa* is the major cause of hospital acquired infections in immunocompetent and immunosuppressed individual such as, burn and surgical sites, UTIs, pneumonia, otitis externa, and soft tissues. Nowadays, the emergence of *P. aeruginosa* isolates that resist β-lactam antibiotics become a serious public health issue globally. The aims of the present investigation are to estimate the prevalence of *Pseudomonas aeruginosa* isolates among patients, to determine their sensitivity and resistance to the prescribed antibiotics in this area and to identify some genes that are responsible for resistance. The study was conducted from April 2021 to March 2022, and included 360 samples taken from patients admitted to three Teaching Hospitals (Azadi, Burn and Heevi) and the Vajeen Private Hospital in Duhok city.

The overall prevalence of *Pseudomonas aeruginosa* was 30% (108/360). The highest rate in patient samples was 48.78% from sputum, followed by nearly equal rates of 38.78% and 38.3% from both of ears and burns, respectively and the lowest rate 19.34% from urine with highly significant (P<0.001) differences between these sources. The 16 S rDNA gene was amplified by PCR in all isolates, each of them produced a distinct band of 956 bps for this gene. The produced amplicons of 16S r DNA were sequenced, 4 sequences were deposited in GenBank under accession numbers: OP221013, OP221014, OP221015, OP221016 from sputum, ear, burn and wounds, respectively. In this study also, two antibiotic resistant genes were detected included $bla_{\text{OXA-10}}$ and bla_{BEL1}. The gene $bla_{\text{OXA-10}}$ was present in100% of the isolates, while the gene bla_{BEL1} was detected in 95.83%. The sequence of $bla_{\text{OXA-10}}$ gene was deposited in GenBank under accession number: OP251042. All isolates carried these genes were 100% resistant to β-lactam antibiotics in addition to Erythromycin and Trimethoprim/Sulfamethoxazole, furthermore about 34% of these genes were detected among ages >50 years.

Keywords: ***Pseudomonas aeruginosa, inpatients, molecular PCR, antibiotic susceptibility***

INTRODUCTION

Pseudomonas aeruginosa is rod-shaped Gram-negative mono-flagellated and non-sporulated bacterium belonging to the family Pseudomonadaceae (Diggle and Whiteley, 2020). This bacterium is responsible for hospital acquired and nosocomial infections including otitis media, soft tissues, cystic fibrosis, urinary tracts, ulcerative keratitis, skin and surgical site infections (del Barrio-Tofino *et al.*, 2020).

Pseudomonas aeruginosa can survive in the hospital environment, in the community settings and is able to resist adverse environmental conditions (Lister *et al.*, 2009). The infections caused by this bacterium are difficult to be treated due to its resistance to numerous antimicrobial agents such as β-lactams, quinolones and aminoglycosides (Pang *et al.*, 2019). This bacterium produces the enzyme β-lactamase that hydrolyze the β-lactams ring which constitute the main mechanisms of intrinsic resistance produced by it. β-Lactamases are of four classes, the classes A, B, and C, possess the active-site serine, while class B is the zinc-dependent or also called metallo-β-lactamases (MBLs; class B). *P. aeruginosa* produce class C β-lactamase that exhibit resistance to antipseudomonal cephalosporin's (Oliver *et al.*, 2015). Numerous *P. aeruginosa* strains produce extended-spectrum-β-lactamases (ESBLs) due to the excessive use or abuse of antibiotics leading to the production of ESBL producing bacteria that are responsible for initiating resistance to β-lactam antibiotics such as cefotaxime, ceftriaxone, ceftazidime, and aztreonam. Most of the ESBLs are belonged to class A, except OXA-type ESBLs are in class D of this enzyme (Pachori *et al.*, 2019). *Pseudomonas aeruginosa* is able to form biofilms in the clinical settings that protect it from host defenses particularly during chronic infections (Ciofu and Tolker-Nielsen, 2019). This bacterium can be isolated from ventilators in the intensive care units and other equipment's in the hospitals (Pang *et al*., 2019; Rocca *et al.*, 2020).

The occurrence of high infections with *P. aeruginosa* isolates that are resistant to antibiotics in the hospitals, encouraged us to conduct this cohort study for determining the prevalence, the antibiotic sensitivity and the identification of some of the antibiotic resistant genes of *P. aeruginosa* among patients in four major hospitals, three teaching hospitals (Azadi, Burn, Heevi pediatric) and the Vajeen Private hospital of Duhok city in order to improve our infection control measures.

1. Materials and Methods

Sample collection:

This study was performed in three teaching hospitals (Azadi, Burn and Heevi pediatric) and one private hospital (Vajeen) in Duhok city, during which 360 samples were obtained from patients, during April 2021 to March 2022. These samples included: 179 swabs (42 wounds, 47 burns, 49 ears and 41 sputum's), and 181 midstream urine samples. All samples were obtained from patients of both genders and different ages (8 months to above 60 years). The samples were collected after taking consents from the enrolled patients or the accompanied parent of the children after giving a clear explanation of the objectives and logistics of the study. The patients who refused to give consent and those who didn't agree to be included in the study were excluded. In addition, an ethical approval to perform the study was obtained from Duhok General Directorate of Health (No.1808 in 31-8-2021). Each collected sample was placed immediately in a clean and sterile container with sterile transporting medium and fully labeled with the patient information according to the designed questionnaire for the study. Finally, all of the collected samples were placed in a cool box and transported to the microbiology laboratory in Azadi teaching hospital for further processing.

Isolation and identification of Pseudomonas aeruginosa

In the laboratory, all of the obtained samples were cultured on MacConkey, Nutrient, Blood and Cetrimide agars and incubated at 37˚C for 24 hours. The produced *P. aeruginosa* colonies were identified according to gram stain, their morphological characteristics on Cetrimide and MacConkey agars in addition to the biochemical tests such as catalase and oxidase tests (Shields and Cathcart, 2010). Each isolate of *P. aeruginosa* was maintained in 10 ml Brain Heart Infusion agar slants and stored at 4˚C to be used for antibiotic susceptibility test (AST).

All confirmed *P. aeruginosa* isolates were stored for long time in 50% glycerol broth at -20 ˚C. This was done by inoculating 3ml of sterile nutrient broth with a small portion of pure isolated colony, then the tube containing the inoculated broth was incubated at 37 ℃. Later on, 0.5 ml from this bacterial growth was added to a sterile tube containing 0.5 ml of sterilized glycerol, mixed well and stored at -20 ℃. The bacteria can be maintained for 6 months in this condition to be used for molecular study (Manjana *et al.,* 2016).

Antibacterial susceptibility test

The susceptibility of *Pseudomonas aeruginosa* was tested by two methods, the VITEK 2 automated photometric systems which have accurate susceptibility testing and detect *P. aeruginosa* at shorter time (Torrecillas *et al.,* 2020). Also, the Kirby-Bauer disc diffusion method using Mueller-Hinton agar according to the Clinical Laboratory Standards Institute for testing the antimicrobial susceptibility (CLSI, 2021).

DNA extraction and PCR amplification

DNA was extracted from *P. aeruginosa* isolates that exhibited resistance to antimicrobial agents using FavorPrep^{TM} Genomic DNA Extraction Kit (Favorgen-Taiwan), depending on the manufacturer's instructions. The concentration and the purity of the yielded DNA was measured using NanoDrop spectrophotometer. The produced DNA then was stored in a deep freezer at -20℃, to be used later for molecular studies. The 16S rDNA, *bla*$_{BEL1}$ and *bla*$_{OXA-10}$ genes were targeted with specific primers using the single plex polymerase chain reaction as shown in Table 1.

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

Name of primer	Sequence (5′---3′)	Molecular weight	References
16S rDNA-F	5′-GGGGGATCTTCGGACCTCA-3′	956 bp	Spilker *et al.,*2004
16S rDNA-R	5′-TCCTTAGAGTGCCCACCCG-3′		
Oxa-10-F	5′-CCGAAGCCGTCAATGGTG-3′		Laudy *et al.,*2017
Oxa-10-R	5′-CCAACCCACCATGCGACA-3′	571 bp	
BEL1-F	5′-GAAACTGCTGCTCTACCCG-3′		
BEL1-R	5′-CGCCTTGCAATTCAGGTGC-3′	788 bp	

The PCR reaction composed from 30 µL included 15 µl of PCR master mix (ADDBIO.INC, South Korea), 4 µL of DNA sample, 7 µl of deionized distilled water, 2 µl of each primer including the forward and reverse primers (10 pmol/µl) to each reaction. Thermocycles PCR was used for amplifying the extracted DNA as illustrated in Table 2.

Table 2. Single plex PCR amplification conditions for *16S r DNA*, *bla*$_{BEl\ 1}$ and *bla*$_{OXA\ 10}$ genes of *P. aeruginosa*

Genes	Temperature (°C)/ Time					
	Initial denaturation	Denaturation	Annealing	Extension	Final Extension	Cycle No
16S rDNA	95 °C 5 mins	94 °C 30 sec	58 °C 30 sec	72 °C 45 sec	72 °C 7 min	35
bla$_{BEL\ 1}$	94 °C 5 mins	94 °C 30 sec	57 °C 30 sec	72 °C 30 sec	72 °C 5 min	35
bla$_{OXA\ 10}$						

The PCR products were run electrophoretically on 1.5 % agarose gel stained with 6 µl of RedSafe dye in Tris-Borate EDTA buffer (10X). The submerged gel was run for 15 minutes at 45 Voltage then the

voltage was raised to 85 Volt for one hour. DNA ladders with 100-1500 bp was used as a reference to determine the molecular weights of the PCR products. The gel was examined under UV Transilluminator to confirm PCR amplification (Maniatis et al.,1982).

Sequences analysis

The PCR products (30 µl) of *P. aeruginosa* isolates were sent to Macrogen Company (Seoul, South Korea) for sequencing using the primers used for *16S r DNA, bla*$_{BEL1}$ and *bla*$_{OXA-10}$. The obtained DNA sequences were analyzed and edited using BLAST and BioEdit software. Then these sequences were submitted to the GenBank database. The gathered data were analyzed statistically using SPSS version 25 software, the Chi-square (X2) test was used to determine the differences between the variables. *P* value ≤ 0.05 was considered significant, and more than this value was considered as non-significant.

2. Results and Discussion

Prevalence of *Pseudomonas aeruginosa* in patients' specimens

The results of the current study illustrated that 30% (108/360) of the patients' samples showed positive bacterial growth of *P. aeruginosa*. Among the clinical samples, the sputum samples exhibited the highest rate of 48.78% of the isolates, followed by nearly equal rates from both of the ear and burn samples (38.78% and 38.3%), respectively. While the lowest rate was isolated from urine samples which was 19.34%. Statistical analysis showed the presence of highly significant (P <0.001) differences between these samples.

Similar findings were reported in Baghdad by Friyah and Rasheed (2018), who recorded the highest rate of *P. aeruginosa* isolates from sputum samples and the lowest from urine samples which were 27.78% and 7.41%, respectively. While in Kirkuk, two studies were performed, in the first the highest rate (40%) of *P. aeruginosa* was isolated from burn patients and the lowest (10%) from urine (Hasan *et al.*,2019). While in the second study, the highest rate of *P. aeruginosa* isolates (74.19%) was from wounds (Hasan *et al.*,2019). The variation in the isolation rates of *P. aeruginosa* among samples from different sources may be attributed to the gender, patient age, to the types and number of the samples, the method used for obtaining them, the season of collection and patient residency (Saderi *et al.*,2019). Table (3) illustrate the prevalence of *P. aeruginosa* among various patient's samples.

Table 3. The Distribution of *Pseudomonas aeruginosa* among clinical specimens

Clinical samples	Number of tested samples	Positive (%)
Sputum swab	41	20 (48.78)
Wound swab	42	16 (38.1)
Ear swab	49	19 (38.78)
Burn swab	47	18 (38.3)
Urine	181	35 (19.34)
Total	360	108 (30)
P value <0.001, X^2 =21.355		

Regarding to the relationships between the gender and age of the patients. Females showed the highest rate as compared to males (37.37% vs 21.76%), and this difference was statistically highly significant (P=0.001) (Table 4). In Suleimani, Othman et al. (2014) reported higher rate of isolates from females than males (57% vs 43%), respectively and in Kirkuk, Hasan et al. (2019) also reported a higher rate of isolates among females than males (52.5 vs 47.5%) respectively. Additionally, they attributed this higher rate in females to the urolithic mucosal adherence to mucopolysaccharide lining or to anatomical predisposition of females in addition to numerous host factors.

The highest rate of *P. aeruginosa* isolates was obtained from ages over 50 years which was 38.96%, while the lowest rate (18.84%) was from ages over 10-20 years. Variable rates of *P. aeruginosa* isolates were recorded among different ages in previous studies. Al-Saffar and Jarallah (2019) in Nasiriya, recorded a rate similar to the current study which was 38.9%, but from younger ages (5-25) years. While, in Kirkuk, the highest rate of 45% was recorded among ages of 15 - 30 years (Hasan *et al.*,2019). The higher rate of *P. aeruginosa* isolates in the elderly patients in the present study might be attributed to more critical underlying diseases in addition to weak immune status at these ages, as at old ages once infection is acquired it is difficult to treat and control and such patients need prolonged treatment and recovery time (Folic *et al.*, 2021).

Table 4: The relationship between the number and rates of *P. aeruginosa* isolates among both genders and various ages

Variables	No. Examined	*P. aeruginosa* No. (%)	*P* value
Gender			
Males	170	37 (21.76)	*P* value=0.001 X^2 =10.402
Females	190	71 (37.37)	
Age			
<1-10	66	20/66(30.30)	
>10-20	69	13/69 (18.84)	
>20-30	59	22/59 (37.29)	*P value* =0.97 X^2 =9.315
>30-40	56	14/56 (25)	
>40-50	33	9/33 (27.27)	
>50	77	30/77 (38.96)	
Total	360	108 (30)	

Antibacterial susceptibility test

All of the 108 isolates of *P. aeruginosa* were tested for their antibiotic susceptibility against fifteen antibiotics that were usually prescribed by physicians as demonstrated in Table (5).

Table 5: Antimicrobial susceptibility pattern of *Pseudomonas aeruginosa* isolates.

	Specimens					Total	Susceptibility
Antibiotics	Sputum No.(20)	Urine No.(35)	Wounds No. (16)	Ears No.(19)	Burns No.(18)	No.(108)	%
Colistin	15	34	14	15	18	96	88.9
IPM	18	27	11	19	10	85	78.7
ATM	18	23	9	16	10	76	70.4
LEV	19	18	9	16	6	68	63
MEM	16	22	8	17	0	63	58.3
CAZ	14	24	9	12	0	59	54.6

CIP	18	18	8	13	0	57	52.8
CN	15	16	6	13	0	50	46.3
AK	15	16	5	13	0	49	45.4
FEP	9	13	5	7	0	34	31.5
CRO	13	14	0	4	1	32	29.6
AM	0	1	0	0	0	1	o.93
AX	0	0	0	0	0	0	0
E	0	0	0	0	0	0	0
SXT	0	0	0	0	0	0	0
Colistin (COL); Imipenem (IPM); Aztreonam (ATM); Levofloxacin (LEV); Meropenem (MEM); Ceftazidime (CAZ); Ciprofloxacin (CIP); Gentamicin (CN); Amikacin (AK); Cefepime (FEP); Ceftriaxome (CRO); Amoxicillin (AM); Amoxicillin clavulanate (AX); Erythromycin (E); Trimethoprim/sulfamethoxazole (SXT).							

Pseudomonas aeruginosa isolates in this study showed the highest susceptibility to Colistin which was 88.9%, indicating that this antibiotic was the most effective antibiotic. Somewhat slightly higher rate to Colistin was reported by Oumeri and Yassin (2021), in Duhok city /Iraq, as they recorded a susceptibility rate of 91.7% for Colistin. This antibiotic act on the outer cell membrane of Gram-negative bacteria by binding to lipopolysaccharides and phospholipids and displaces Ca^{+2} and Mg^{+2} cations from the phosphate group of lipids. Therefore, can cause cell membrane disruption and intracellular contents leakage resulting in cellular death (Bialvaei and Samadi, 2015). The second effective antibiotic was Imipenem, that showed a susceptibility rate of 78.7%. Previous studies in Duhok also, reported higher susceptibility rates of 87.3% and 95.2%, respectively to Imipenem (Al-Derzi,2012; Thalhammer and Hörl, 2000). Imipenem is a potent cell wall synthesis inhibitor; its therapeutic effects are due to crossing the cell wall through porins and it binds to penicillin binding proteins in the bacterial cell membrane (Tsao *et al.*,2018).

Aztreonam, was the third effective antibiotic in the current study with a susceptibility rate of 70.4%, this antibiotic was not studied previously. Like other β-lactam antibiotics, Aztreonam inhibits bacterial cell wall synthesis because it possesses a high affinity for penicillin binding protein 3(PBP3). Therefore, it inhibits the 3rd and last step of bacterial cell wall synthesis causing the lysis of the cell wall which is mediated by cell wall autolytic enzymes (Finberg and Guharoy, (2021). The antibiotic with the lowest susceptibility was Meropenem with a rate of 58.3%. This rate is in accordance with the rates obtained by Yassin *et al*. (2014) and Al-Derzi(2012) in studies performed among immunocompromised patients. Tam *et al*. (2005) suggested that Meropenem is less susceptible than other β-lactams to the inoculum effect. With respect to Aminoglycoside (Gentamicin and Amikacin) they exhibited moderate rates of susceptibilities of 46.3% and 45.4 %, respectively. Such results are consistent with a previous study of Al-Derzi (2012).This effect is attributed to the inhibition of the bacterial protein synthesis by Aminoglycoside antibiotics because they binds to ribosomal 30S subunits (Mingeot-Leclercq *et al.*,1999).

The high resistant rate of *P. aeruginosa* isolates to various antibacterial agents among nosocomial organisms is considered as a serious challenge in the treatment of this bacterium which is mainly attributed to the wide use of antimicrobial agents as stated by WHO (2002). Thus, creating a critical situation for the spread of resistant bacterial strains by involving variable resistance mechanisms including the production of β-lactamases that destroy these drugs. Additionally, this bacterium produce biofilm that enable it for long survival in hospital units and to acquire the genes responsible for extensively drug resistance from the hospital environment. This situation, poses threat to patients that may be difficult to treat when infected with such infections. The hospital surfaces, the hands of the hospital staffs and visitors can be contaminated with this bacterium and from these sources it can spread to the patients and to the community. Therefore, antibiotic susceptibility test is important for *P. aeruginosa* treatment because this bacterium causes many

infections in addition to its resistance to many antibiotics (Yoshimura and Nikaido, 1982). Nonetheless, antibiotic resistance patterns are known to change over time and vary significantly according to environment and infection type (Khoshnood *et al.*, 2019).

Molecular Identification of Pseudomonas aeruginosa using 16S rDNA

PCR amplification of the *16S rDNA* was done to detect *Pseudomonas aeruginosa*. The size of the produced amplicon for *16S rDNA* was 956 bp and the produced amplicons of *16S r DNA* were sent to south Korea for sequencing and the received sequences were submitted to GenBank and recorded under accession numbers: OP221013, OP221014, OP221015, OP221016 from sputum, ear, burn and wounds, respectively, except those from urine specimens for which accession numbers were not recorded (Figure 1).

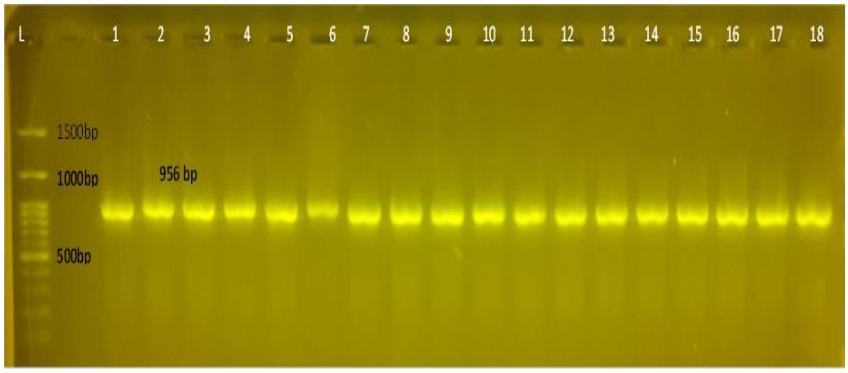

Figure (1): The PCR amplification of the 16s rDNA gen used for *P. aeruginosa* identification with a molecular weight of 956 bps, using 1.5 % agarose gel electrophoresis. Lane L contained DNA marker with a molecular weight of 100-1500 bps, lanes (1-24) showing the specimen's bands.

Molecular Detection of Antibiotic Resistant Genes.

Using PCR amplification, the prevalence of the antibiotic resistant genes ***bla*OXA-10 and *bla*BEL1** among *P. aeruginosa* isolates were examined and confirmed by agarose gel electrophoresis. Among the ESBL genes, the *bla*$_{\text{OXA-10}}$ gene was detected in all of the 24 isolates with a molecular weight of 571 bps, as shown in Figure (2). The analyzed isolates and the amplified sequence for *bla*$_{\text{OXA-10}}$ was recorded in GenBank under accession number: OP251042.

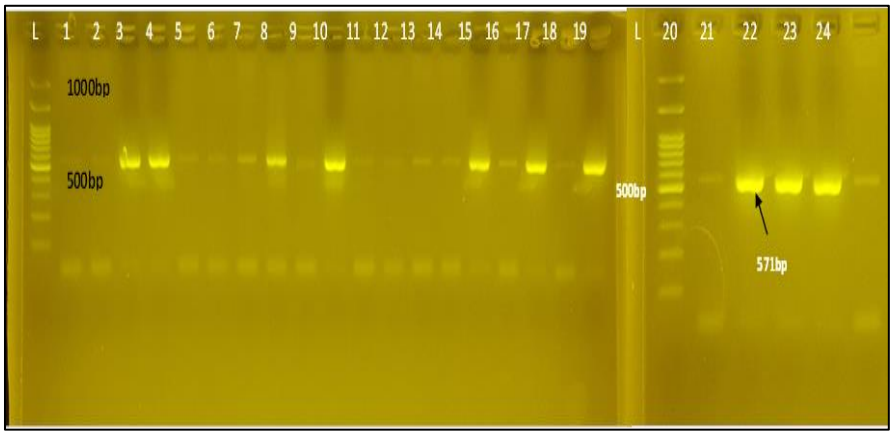

Figure (2): The PCR amplification of the *bla*$_{\text{OXA 10}}$ gene of *P. aeruginosa* with a molecular weight of 571 bps, using 1.5 % agarose gel electrophoresis. Lane L contained DNA marker with a molecular weight of 100-1500 bps, lanes (1-24) showing the specimen bands.

On the other hand, *bla*$_{BEL1}$ gene was present in *P. aeruginosa* with 788 bps. It was amplified in 95.83% (23/24), using species-specific primer, except one of the wound isolates (no.6) was negative for this gene as shown in Figure (3).

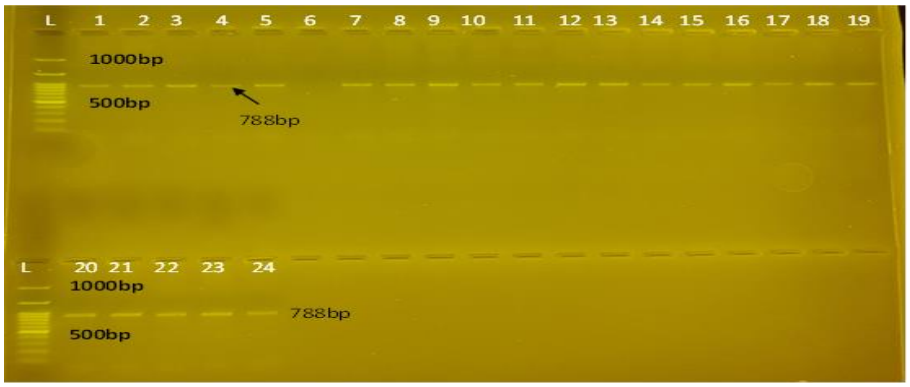

Figure (3): The PCR amplification of the *bla*$_{BEL1}$ gene for *P. aeruginosa* identification with a molecular weight of 788 bp, using 1.5 % agarose gel electrophoresis. Lane L contained DNA marker with a molecular weight of 100-1500 bps, lanes (1-5, 7-24) showing the specimen's bands.

This gene belongs to class D classified according to amino acid base action, it hydrolyzes amino acids, and spread among nosocomial *P. aeruginosa* infection, it was detected in all analyzed isolates (100%). The available data indicated that *bla*$_{OXA-10}$-ESBLs is encoded by plasmid. (Senbayrak *et al.*,2015). In a recent study in Zakho city this gene was detected in 59.26% of ESBL-producing isolates from burn patients (Polse *et al.*,2023). While a much lower rate (38.2%) of this gene was detected among patient specimens in Erbil city hospitals (Ganjo and Mansoor, 2020). In Iran also, a low rate of 54.16% for *bla*$_{OXA-10}$ was recorded by Farshadzadeh *et al*. (2014) using the same primers of the current study. While a study performed in Shiraz city, Iran reported a rate of 76.2% *bla*$_{OXA-10}$ (Emami *et al.,* 2015).

This study presents a deep insight on the high occurrence of resistant genes among the analyzed *P. aeruginosa* isolates from various clinical specimens of patient which are resistant to diverse types of antibiotics in Duhok city. This is a critical situation which necessitate the minimization of their spread, prevent outbreaks and to optimize the treatment. Therefore, it's advisable to monitor the antimicrobial resistance and disseminate strict compliance toward infection control programs to reduce the spread of multidrug resistant microorganisms among the community.

The gene *bla*$_{BEL1}$ (*Bel 1*) with 788 bps, was present in 95.8% of *P. aeruginosa* isolates, except one wound isolates. Also, this gene is one of the resistant factors that is established in *P. aeruginosa* and produced during the course of clinical infections, *bla*$_{BEL1}$ hydrolyze most of extended-spectrum antibiotics such as Aztreonam, Cephalosporins, Tazobactam and Imipenem which are affected by inhibition of Clavulanic acid and is chromosome encoded ESBL gene belongs to class 1 integrons composed of three other genes (Rosa *et al.*,2019).

3. **Conclusion:**

These findings will be helpful to advise treatment with appropriate antibiotic strategy against multi- and extensively drug -resistant *P. aeruginosa* to cope with the chances of evolving resistant pathogens.

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