Evaluation of blaGES-5 and bla veb-1 genes with multidrug-resistant extend, pandrug resistance patterns (MDR, XDR, PDR), and biofilm formation in Pseudomonas aeruginosa isolates

Fattma A. Ali, Sevan Hassan Bakir, Sayran Hamed Haji, Bashdar M. Hussen

1 College of Health Sciences, Hawler Medical University, Erbil, Kurdistan Region-Iraq
2 Medical Laboratory Technique, Erbil Medical Technical Institute, Erbil Polytechnic University, Erbil, Kurdistan Region-Iraq
3 College of Pharmacy, Hawler Medical University, Erbil, Kurdistan Region-Iraq

Abstract: Pseudomonas aeruginosa is a ubiquitous microorganism that is difficult to treat due to the increasing prevalence of multidrug resistance patterns. A total of 227 samples were taken from different clinical samples during the study period from January 2018 to December 2018. The isolates were identified with antibiotic sensitivity testing with ESBL by the Vitek-2 automated method. MDR, XDR, and PDR were determined. 40 (17.6%) isolates were positive for P. aeruginosa, maximum of ESBL and MBL enzymes in 21 (52.5%) of our isolates. Imipenem followed by Meropenem were found to be effective against ESBL and MBL producers. Resistance was reached between 72-100% to 5 antibiotics. The frequency of PDR, MDR, and XDR were 5%, 50%, and 45%, respectively. The frequency of co-production between MDR, XDR, and PDR with MBL, ESBL, and Biofilm was 35%, 12.5% and 5%, respectively. Among the ESBLs, the frequency of distribution of bla veb-1 gene and bla GES-5 gene was 50% and 40%, respectively. Bacterial isolates simultaneously carrying bla veb-1 gene with multiple β-lactamases of different classes of biofilm, MDR, PDR, and XDR as same as a coexisting bla GES-5, gene. One isolate was detected as new isolates registered in global gene bank as locally P. aeruginosa isolates in Erbil city (LOCUS MN009053). The phylogenetic trees of the bla veb-1 gene isolates were demonstrated a genotype closely related to others, deposited in GenBank similar to the P. aeruginosa gene; gene sequencing revealed a 99% similarity with other isolates deposited in GenBank.

Key words: Pseudomonas; XDR; PDR; Biofilm; blaGES-5; bla veb-1.

Introduction

Pseudomonas aeruginosa is the major cause of nosocomial infection in medical settings, bringing about severe infection in patients with background diseases (1). For such infections, antimicrobial therapy may become a difficult task because P. aeruginosa is resistant to many antibiotics and even develops resistance to several antibiotics during treatment. In this context, infections of P. aeruginosa that demonstrate acquired resistance to beta-lactam drugs are one of the most challenging targets for antimicrobial therapy and are responsible for high rates of therapeutic failure, increased mortality, morbidity, and overall cost of treatment (2). Those strains of P. aeruginosa that are resistant to at least one or more of the three or more classes of anti-pseudomonal antibiotics are called multi-drug resistant (MDRs). The term extremely drug-resistant (XDR) refers to those isolates that are resistant to at least one antibiotic agent from six or more of the anti-pseudomonal antimicrobial groups. If a P. aeruginosa isolate is resistant to all anti-pseudomonal antimicrobials, it is called Pandrug resistant (PDR)(3). ESBLs are a group of enzymes produced by certain microorganisms with the ability to hydrolyze a broad range of β-lactams, including penicillins, cephalosporins, and aztreonam, whereas cannot hydrolyze cephamycins and carbapenems (i.e., Imipenem) professionally. In general, ESBLs are not codified by the bacterial chromosome but are usually codified by transferable plasmids. The transferable plasmids can harbour many different genes and have the ability to transmit a copy of themselves to other bacteria (4). These enzymes are a major concern regarding the future of antimicrobial chemotherapy because of their rapid transition among microorganisms. Microbial biofilm is a structure of the microbial community that is found attached to living or non-living surfaces. These three-dimensional structures are always enclosed within a complex matrix. Biofilm-producing microorganisms — compared to non-producing counterparts — are often more resistant to antimicrobial agents; so that in some cases, three to four times the concentration of antibiotic compounds is required to kill biofilm-producing microorganisms, depending on the antibiotic composition used (5). Various mechanisms including delayed penetration of the antimicrobial agents through the biofilm matrix, the altered growth rate of biofilm organisms, and other physiological changes endow resistance to biofilm-forming microorganisms (6). Thus, biofilm formation paves the road for pathogen’s survival and persistence under unfa-
favorable conditions like host invasion or following antibiotic treatment (7). The epidemic prevalence of MDR / XDR strains in the healthcare setting has always been a concern of pathologists. In recent years, evidence of MDR / XDR spread in various hospitals across the world has been reported, indicating the presence of high-risk clones (8). Early detection of gram-negative bacteria such as P. aeruginosa, which produces MBL and ESBL, is of particular importance to medical centers to prevent the spread of these strains and to provide appropriate medical services to hospitalized patients and patients with acute illness (9). In P. aeruginosa, five types of acquired class A ESBL were identified, VEB-, PER-, GES, TEM- and SHV. OXA type ESBLs belong to molecular class D. VEB, PER, and OXA are the most predominant ESBL types reported in P. aeruginosa. The molecular classification of β-lactamase bacteria relies on the sequence of nucleotides and amino acids in these enzymes (4). bla_{VEB} (for Vietnamese extended-spectrum beta-lactamase), bla_{GES} has the highest amino-acid similarity with Pseudomonas Extended Resistance (PER-1) and (PER-2) (38%), which confers remarkable resistance to Ceftazidime, Cefotaxime and Aztreonam (10). Genes encoding several enzymes that cause resistance to Beta-lactam antibiotics are classified in Class A which includes but is not limited to bla_{VEB} and GES enzymes encoding genes. ESBL are enzymes produced by Gram-negative bacteria that are encoded by plasmid gene sequences (11). The bla_{VEB,1} gene has been reported in several enterobacterial isolates from Southeast Asia (12) and P. aeruginosa clinical isolates (13), GES-type β-lactamase was originally identified as a cephamycin-hydrolysing extended-spectrum β-lactamase family. Twenty-six variants of the GES group have been identified, some of which are classified as carbapenemases (14) bla_{GES} have been reported worldwide as a carbapenemase harbored Klebsiella pneumoniae, Escherichia coli, and P. aeruginosa (15). Prior to the outbreak described in this report, only GES-3 and GES-4 had been detected in Japan (in K. pneumoniae). The MDRP of the initial case was either acquired within the community or imported from abroad. As this is the first report of GES-5 detection in Japan, we will be able to monitor the possible spread of GES-5 in the surrounding area. Molecular detection and monitoring of resistance genes are of great importance to establish appropriate antimicrobial therapy and to evaluate and monitor the spread of drug-resistant P. aeruginosa. This research was carried out to determine the presence of blavb-1 and blages-5 among ESBL and MBL production and biofilm formation among MDR, XDR, and PDR producing P. aeruginosa isolated from different clinical samples (16).

Materials and Methods

Isolation and identification of bacteria

This present study was carried out in Rizgary, Teaching hospital, Laboratory center, Raparin, Nanakaly hospitals in Erbil province at a period from (September 2016 – March 2017). A total of 227 samples (sputum, urine, wound, burn, Bronchial wash) were collected and inoculated onto Blood Agar and MacConkey Agar plates. Plates were incubated at 37°C for 24-48 hours under aerobic conditions. Provisional identification of P. aeruginosa was performed based on microscopic properties, culture characteristics, biochemical tests, definitive identification by Vitek 2 Compact: colorimetric technology for bacterial identification; gram-negative (GN) card was used for P. aeruginosa identification. All the following steps were done according to the manufacturer’s instructions (Biomerieux, France). The Ethics Committee approved the study of Hawler Medical University/COLlege of Health Sciences.

Antimicrobial susceptibility test

In vitro susceptibility testing of all collected isolates was performed using the VITEK 2 compact system, an automated ID and susceptibility (AST) system for a wide range of antimicrobials. VITEK 2 system includes an Advanced Expert System (AES) susceptibility test that typically uses different antimicrobial susceptibility test cards (AST cards) according to the pathogens predicted. The related cards were inoculated and incubated in the system, as instructed by the manufacturer. The results of the VITEK 2 compact system susceptibility test were obtained as MIC values. They demonstrated as susceptible, the intermediate or resistant breakpoint according to the National Clinical Laboratory Standard Committee (17). Final results were represented using the Advanced Expert System (AES) that is the experiment was repeated wherever indicated by the AES (18). Multidrug-resistant (MDR, strain resistant to at least one antibiotic out of three or more antimicrobial classes), extensively drug-resistant (XDR, resistant to at least one antibiotic in all but two or fewer antimicrobial classes (i.e. bacterial strain shows susceptibility to only one or two categories), (PDR, resistant to all antibiotic classes) were determined based on the guideline of European Centre for Disease Prevention and Control (ECDC), and Centers for Disease Control and Prevention (CDC).

Phenotypic screening for ESBL

VITEK 2 system equipped with the antimicrobial susceptibility test extends AST-EXN8 card was used to test all isolates of P. aeruginosa. This system was designed to perform both screening and confirmatory tests for phenotypic detection of ESBL on the same plate. The test comprises a panel of six wells containing ceftazidime 0.5 mg/L, cefotaxime 0.5 mg/L, and cefepime 1.0 mg/L; the same antibiotics in combination with clavulanic acid (4, 4, and 10 mg/L, respectively) were poured in the other three wells. An optical reader was used for the quantitative measurement of bacterial growth. The reduced bacterial growth in wells containing Cephalosporin/clavulanate compared to those only containing the cephalosporin was regarded as the indicator of ESBL production. All phenotypic interpretations of ESBLs were reported as positive ESBL screening results. Strains were reported as ESBL-negative whenever phenotypic interpretations other than ESBLs were proposed by the AES (18).

Metallo beta lactamase

The double disk approach was used to detect MBL production in P. aeruginosa isolates. Bacterial colonies were suspended in Mueller-Hinton broth; the suspension turbidity was calibrated to equal 0.5 McFarland stan-
 Phenotypic screening of biofilm formation

Congo red agar method

*P. aeruginosa* suspension was inoculated on a specially formulated solid medium (BHI) with 5% sucrose and Congo red. The medium consisted of BHI (37 gms / L), sucrose (50 gms / L), agar no.1 (10gms / L) and red stain (0.8 gms / L) from the Congo. Red Congo was prepared as a condensed water solution and autoclaved for 15 minutes at 121 °C. The agar was then applied separately from the other medium constituent when it had cooled to 55 °C. Plates were inoculated and aerobically incubated at 37 C for (24-48) hours. Black colonies with solid, crystalline consistency showed a good outcome. Weak slime producers generally remained pink, while occasional darkening was observed in the center of the colonies. An indeterminate result was an obscuring of the colonies with the lack of a dry crystalline colonial morphology. The triplicate experiment was conducted and replicated three times (20).

Detection of *bla* 

Polymerase chain reaction (PCR) using specific primers (Table 1) was performed to detect *bla* _GES_ and *bla* _VES_ genes. Total DNA was extracted from the isolates using a DNA extraction kit (Bioneer, Daejeon, Korea). PCR procedure was conducted in a thermocycler (Applied Biosystems, Carlsbad, CA, USA) as follows: 96 °C for 5 min; 35 cycles of 1 min at 96°C, 1 min at a specific temperature for each primer, and 1 min at 72°C; and a final extension step of 10 min at 72 °C. Amplification mixture was prepared in a total volume of 25μl including 12.5μl Taq DNA polymerase 2× Master Mix with 1.5mM MgCl₂ (Ampliqon, Odense, Denmark), 0.5μM of both forward and reverse primers, 9μl nuclease-free water, and 2.5μl DNA template (50pg concentration). PCR products were electrophoresed on a 1% agarose gel at 100V, stained with ethidium bromide solution, and finally visualized with a gel documentation system (UviTec, Cambridge, UK).

DNA Sequencing

PCR products were sent to National Instrumentation Center for Environmental Management for Sequencing (Seoul, Korea). *bla* _VES_ sequences were compared with those available in the GenBank database (National Center for Biotechnology Information, Bethesda, MD, USA) through BLAST searches. Sequence alignment was performed with closely related reference sequences from other *P. aeruginosa* isolates, available in GenBank, using the BLAST (http://www.ncbi.nlm.nih.gov). A neighbor-joining tree with combined *bla* _VES_ data was constructed, according to the maximum likelihood method, using Molecular Evolutionary Genetics Analysis (MEGA) 7 software (ver. 7, Pennsylvania State University, State College, PA, USA) (21).

Statistical analysis

Statistical Package for Social Sciences (SPSS) software, version 22 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Fisher exact test or chi-squared test was performed in order to analyze categorical data. A P value of <0.05 was considered statistically significant.

Results and discussion

*P. aeruginosa* represents a major cause of acute nosocomial infections, particularly in critically ill and immune-compromised patients. Indeed, *P. aeruginosa* is the top pathogen causing ventilator-associated pneumonia and burn wound infections and is a major cause of nosocomial bacteremia, with a very high (>30%) associated mortality rate (22). Out of 227 samples investigated in this research, 40 (17.6%) isolates were positive for *P. aeruginosa*, as shown in Figure 1. It was observed that the most incriminating in burn infection account for (2.6%) of the total number of isolates, the results obtained agreed somewhat with the findings of (23), who found *P. aeruginosa* the most commonly cultured organism (54.2%) isolated from mild to severe form of external otitis and chronic suppurrative otitis media. In contrast, 16% and 46.3% prevalence of *P. aeruginosa* obtained in wound swabs investigated was not similar to the findings of the present study. In contrast, Fifty-four clinical isolates of *P. aeruginosa* were collected from Selayang Hospital, Selangor, Malaysia most isolates thirty-six percent were identified from pus followed by

**Table 1. Primers used for detecting ESBL-encoding genes in this study.**

| Primer | Target Sequence (5'-3') | Annealing temperature (°C) | Reference |
|--------|-------------------------|-----------------------------|-----------|
| VEB-F  | CGACATCCATTTCGATGCGACTCT | 55                          | Aubert et al.,2001 |
| VEB-B  | GCAACAAATAACGC          |                             |           |
| GES-A  | ATGCCCTTCTTCAACGACCTATTGTC | 53                          | Kanayama et al.,2016 |
| GES-B  | CGTGCTCAAGG            |                             |           |
Biofilm production is a major property of *P. aeruginosa* enabling the bacterium to cause chronic infections. The remarkable trait of a mature biofilm is the formation of an extracellular matrix and increased resistance to antibiotics (31). In our study, 75% of the isolates were beta-lactamase producers, ESBL (29). We found coexisting between MBL and ESBL and biofilm-positive isolates mostly occurred in burn samples (10 (25%) for each. As observed, coexisting between MBL and ESBL and biofilm-positive strains (10(25%) did significantly not vary higher.

Although most of the ESBL and MBL producers were resistant to antibiotics, as in Table 3, the association was not statistically significant (*P > 0.05*). Both Imipenem and Meropenem were found to be effective against ESBL producers. In contrast, a statistically significant association (*P< 0.05*) was established between MBL production and antibiotic resistance, and the resistance reached between 72 and 100% to 5 antibiotics; the finding agrees with the study of (35). Besides, various studies have demonstrated MBL producers as pan drug-resistant strains signaling an alarming threat (36). No significant difference was observed between antibiotic susceptibility profiles in biofilm-positive and biofilm-negative in isolates of *P. aeruginosa*. The association between the potential to form strong biofilms by *P. aeruginosa* and antibiotic resistance has also been reported elsewhere (37). The correlation between biofilm formation and antibiotic resistance was significant for aminoglycosides, fluoroquinolones, cephalosporins, imipenem, and pipercillin. Higher antibiotic resistance

respiratory tract (22%) and urine (18.51%) (24). This slight difference can be attributed to geographical factors and also the duration of the experiment.

In our findings were found 29 (72.5%) as MBLs producers of *P. aeruginosa* as in Table 2 higher than a study (23.62%) done by Kamble, 2015 (25), and higher than a study done in India by Agrawal et al (26) which showed only 8.05%. A study conducted in India, Brazil and Iran revealed 32.9%, 30%, 48.3% MBL production respectively (27,28) reported that the percentage of metallo-β-lactamase producers of *P. aeruginosa* was 68%. It is a nosocomial pathogen of particular clinical concern not only because of its extraordinary resistance mechanisms but also for its formidable ability to adapt very well to the hospital environment. In our finding, 80% ESBL production by *P. aeruginosa* was observed and corroborated with a study in Egypt where 95% of *P. aeruginosa* isolates were beta-lactamase producers, ESBL producing *P. aeruginosa* isolates were mostly detected in clinical isolates in a hospital set up according to the university hospital microbiology report. ESBL (29). We found coexisting ESBL + MBL enzymes in 21(52.5%) of the isolates, which was higher than that reported by (30) in which 14.36% of the isolates co-produced ESBL and MBL enzymes.

Biofilm production is a major property of *P. aeruginosa* enabling the bacterium to cause chronic infections. The remarkable trait of a mature biofilm is the formation of an extracellular matrix and increased resistance to antibiotics (31). In our study, 75% of the isolates were able to form biofilms under in vitro conditions, which is lower than 95% reported by (32). (33) showed the high potential of biofilm formation by clinical isolates of *P. aeruginosa* regardless of the sample source. In this research, 43.5% of *P. aeruginosa* burn isolates formed biofilms. Production of ESBL, MBL by non-biofilm and biofilm producers is presented in Table 2. Despite the maximum ESBL producers being biofilm positive, no significant association (*P > 0.05*) was found between isolates, which is similar to the findings reported by (34). Furthermore, there was no significant association between MBL production and biofilm formation. Among the biofilm-producing isolates, 10 (25%) were coproduction with ESBL and MBL producers, Production of MBL and ESBL in biofilm- isolates mostly occurred in burn samples 10 (25%) for each. As observed, coexisting between MBL and ESBL and biofilm-positive strains 10(25%) did significantly not vary higher.

### Table 2. Coexisting between antimicrobial-resistant of ESBL and MBL production and biofilm formation in isolates of *Pseudomonas aeruginosa*

| Sample          | Biofilm N=32 | Biofilm N=29 | Biofilm N=11 | Biofilm N=30 | Biofilm N=10 | Total resistant N=40 |
|-----------------|--------------|--------------|--------------|--------------|--------------|---------------------|
| Burn            | 14(35%)      | 10(25%)      | 7(17.5%)     | 10(25%)      | 4(10%)       | 10(25%)             |
| Wound           | 9(22.5%)     | 8(20%)       | 8(20%)       | 6(15%)       | 2(5%)        | 5(10%)              |
| Urine           | 7(17.5%)     | 7(17.5%)     | 5(6.25%)     | 6(15%)       | 1(2.5%)      | 5(10%)              |
| Sputum          | 6(15%)       | 3(7.5%)      | 5(6.25%)     | 3(7.5%)      | 3(7.5%)      | 3(6%)               |
| Bronchial aspirate | 4(10%)    | 1(2.5%)      | 0            | 3(7.5%)      | 0            | 5(10%)              |
| Total           | 40(100%)     | 29(72.5%)    | 21(52.5%)    | 30(75%)      | 10(25%)      | 50(100%)            |

### Table 3. Relation between antibiotics and different types of beta-lactamase and biofilm.

| Antibiotics | ESBL producers N=32 | ESBL N=29 | MBL producers N=29 | MBL non-producers N=11 | Biofilm Producers N=30 | Biofilm non-producers N=10 | Total resistant N=40 |
|-------------|----------------------|----------|---------------------|-------------------------|-------------------------|---------------------------|----------------------|
| Penicillin  | 32(100%)             | 8(100%)  | 29(100%)            | 11(100%)                | 30(100%)                | 10(100%)                  | 40(100%)             |
| Amikacin    | 25(65.5%)            | 4(50%)   | 21(72.4%)           | 8(72.7%)                | 22(73.3%)               | 7(70%)                    | 29(72.5%)            |
| Gentamycin  | 8(25%)               | 2(25%)   | 10(34.4%)           | 0                       | 9(30%)                  | 1(10%)                    | 10(25%)              |
| Pepracillin | 32(100%)             | 8(100%)  | 29(100%)            | 11(100%)                | 30(100%)                | 10(100%)                  | 40(100%)             |
| Tetracyclin | 6(18.7%)             | 1(12.5%) | 7(24.1%)            | 0                       | 6(20%)                  | 1(10%)                    | 7(17.5%)             |
| Ciprofloxacin| 0                    | 8(100%)  | 8(27.5%)            | 0                       | 7(23.3%)                | 1(10%)                    | 8(20%)               |
| Azithromycin| 0                    | 8(100%)  | 8(27.5%)            | 0                       | 7(23.3%)                | 1(10%)                    | 8(20%)               |
| Cefotaxime  | 0                    | 8(100%)  | 8(27.5%)            | 0                       | 7(23.3%)                | 1(10%)                    | 8(20%)               |
| Chloramphenicol | 32(100%)   | 8(100%)  | 29(100%)            | 11(100%)                | 30(100%)                | 10(100%)                  | 40(100%)             |
| Rifampin    | 32(100%)             | 8(100%)  | 29(100%)            | 11(100%)                | 30(100%)                | 10(100%)                  | 40(100%)             |
| Imipenem    | 2(6.25%)             | 2(25%)   | 2(6.8%)             | 0                       | 1(3.3%)                 | 1(10%)                    | 2(5%)                |
| Meropenem   | 8(25%)               | 2(25%)   | 10(34.4%)           | 0                       | 9(30%)                  | 1(10%)                    | 10(25%)              |
in biofilm producers compared to non-producers has been reported in many studies (36).

The present study showed 20(50%) frequency of MDR P. aeruginosa as presented in Table 4. In comparison (38) reported a 22.7% incidence in Islamabad; while 29% prevalence of MDR P. aeruginosa was found by the study conducted in Peshawar (39) reported. The overall prevalence of antibiotic-resistant P. aeruginosa increases, with up to 10% of global isolates found to be multidrug-resistant (40).

P. aeruginosa (PDR) strains are the most challenging organisms for clinicians to be overcome. According to the results obtained in the present study, 2 (5%) of the total isolates were PDR, which is lower than that reported in Nepal (41). In a study carried out in a tertiary care hospital in Nepal, PDR was also isolated from the clinical samples. This indicates the alarming situation of MDR pseudomonal infections in ICUs of various parts of Nepal (42). Out of 66 isolates, 6.1% were PDR isolates; this sort of complete (or almost complete) lack of treatment options is an increasingly common and desperate occurrence in tertiary care medical centers. The resistance pattern is increasing in such a way that the evolution of PDR P. aeruginosa occurred (43). XDR percentage in the present research (45%) was much more than reported by (44) who reported the value of 36% to this value, validated that the frequency of MDR/XDR P. aeruginosa is certainly on the rise worldwide. Although important geographical differences exist (45), the prevalence of MDR P. aeruginosa has increased in the past decade and currently ranges from 15% to 30% in many areas (46).

On the other hand, in our investigation indicates that burn samples were exhibited the majority of the three types of resistance 6 (42.8%) MDR and 6 (42.8%) XDR, and 2 (14.2%) PDR. It has been reported that patients suffering from background infections such as blood stream infection (septicemia), bronchial infection or diabetic foot ulcers are more susceptible to MDR/XDR P. aeruginosa strain; whereas patients with other types of infection (urine, sputum, ear swabs, eye, and vagina) were 5 times less likely to be infected by an MDR/XDR strain. In this sense, patients treated at the Intensive Care Unit were 3.4 times more likely to become infected by an MDR/XDR P. aeruginosa strain (47).

In addition, interesting results were obtained in the present study regarding co-production of MDR, XDR and PDR with MBL&ESBL&Biofilm 14 (35%), 5 (12.5%), 2 (5%) respectively in clinical isolates. (10%) of MBL producers in our study were MDR and resistant to most of the antibiotics used as in Table 5. A similar result was obtained by (48). This may be attributed to the fact that MBL genes are often carried along with other resistance genes resulting in multi-drug resistance limiting treatment options (Table 5, 6) (49).

The rising antibiotic resistance against commonly used drugs is of great concern. PCR assay was performed to reveal the prevalence of ESBLs genes among 40 isolates of P. aeruginosa (bla<sub>GES-5</sub> and bla<sub>VEB-1</sub>) genes as in Table 6 with amplified size bla<sub>GES-5</sub> 642pb, bla<sub>VEB-1</sub> 642pb as depicted in Figure 2.

Among the ESBLs, the frequency of distribution of bla<sub>VEB-1</sub> gene was 20(50%) and 16(40%) for bla<sub>GES-5</sub> gene, respectively. Maximum bla<sub>VEB-1</sub> positive isolates of P. aeruginosa were observed among burn samples, and maximum bla<sub>GES-5</sub> positive isolates were detected in burn also. In the study by (50) in Tehran, the frequency of the bla<sub>VEB-1</sub> gene was 100% while (51) also in Tehran observed that the frequency of bla<sub>VEB-1</sub> was 31.34%. Contrary to this value, bla<sub>GES-5</sub> genes with 13.3% prevalence have
been reported in another study (52). Various studies have reported *P. aeruginosa* carrying *bla*<sub>GES</sub> gene (53). Another research conducted in Saudi Arabia confirmed the presence of genes encoding *bla*<sub>GES</sub> and *bla*<sub>GES</sub> among extended-spectrum *P. aeruginosa* clinical isolates from burn patients’ (54) Molecular characterization of *bla*<sub>veb-1</sub> like genes from several *P. aeruginosa* isolates showed variability among the *bla*<sub>veb-1</sub> like encoded proteins, as has been found recently in *P. aeruginosa* isolates from Kuwait (*bla*<sub>veb-1</sub> and *bla*<sub>veb-1.b</sub>) (13) 15 strains were isolated from burn units environment *bla*<sub>veb-1</sub> was found in 6 (40%) isolates (4) among those isolates, two isolates (4.6%) produced the ESBL GES-5, possessing the ability to hydrolyze imipenem isolated from blood in Brazil (55).

It is believed that the clinical isolates with (*bla*<sub>veb-1</sub> and *bla*<sub>GES</sub>) genes are associated with the MDR phenotype. Bacterial isolates simultaneously carry multiple β-lactamases of different classes, such as the ESBL20(50%) and MBL 17(42.5%) with biofilm 17(42.5%) and MDR 20(50%) and XDR 2(5%) with gene coexistence and simultaneously encoding ESBLs VeB-1 gene as in Table 7, coexpressing *bla*<sub>GES</sub> gene also with different classes, ESBL 16(40%) and MBL 13(32.5%) with biofilm 16(40%) and MDR 16(40%) and XDR 9(2.25%) and PDR 2(5%) as seen in Table 8. An experiment in Egypt, (9) reported *bla*<sub>veb-1</sub> was 10.4% in *P. aeruginosa* with ESBLs and MBLs in 122 *P. aeruginosa* isolates; but another study (56) revealed that the frequency of MDR and ESBL-positive of the isolates was 19/116 (16.37%), and 8/116 (6.89%), respectively, and 4 isolates (3.4%), amplified *bla*<sub>veb-1</sub>, on the other hand, the production of *bla*<sub>GES</sub> has been shown to be related to expanded spectrum cephalosporin resistance (57). An analysis performed in a hospital in Riyadh, Saudi Arabia (between January to April 2010) indicated that 25 (16%) isolates of *P. aeruginosa* were ESBL producers, with 5 (20%) carrying blaGES genes (58).

The first report in Iran (1) declared that the frequency of *bla*<sub>veb-1</sub> and *bla*<sub>GES</sub> among the ESBL isolates were 24%, and 0%, respectively. In another study, in contrast with the present study, the results of ESBLs genes detection showed that 78.6% of ESBLs producer of *P. aeruginosa* strains carried *bla*<sub>GES</sub> gene while *bla*<sub>veb-1</sub> genes appeared in 10.7% (59). Also, the prevalence of ESBL was 69.44%, and MBL was 42.85% with *bla*<sub>veb-1</sub> 68% and *bla*<sub>GES</sub> 20% as ESBLs in *P. aeruginosa* in Saudi Arabia (54) which was higher than our results. *bla*<sub>GES</sub> was described originally in multidrug-resistant *P. aeruginosa* isolates collected from a South African teaching hospital, were associated with a nosocomial outbreak and displayed expanded hydrolysis to carbapenems, the simultaneous production of ESBLs and MBLs by the same isolate is known to further enhance resistance (13). *P. aeruginosa* XDR clinical isolate co-expresses an MBL and ESBL gene *bla*<sub>GES</sub> in Peru (60), which confirmed our results. The genotype of human bacteria is important in causing a variety of abnormalities and cancers (61-65). Accordingly, research in this regard is valuable and necessary.

To the best of our knowledge, this is the first report of MDR *P. aeruginosa* clinical isolate that co-expressed an ESBL (*bla*<sub>veb-1</sub> and *bla*<sub>GES</sub>) genes with MBL and XDR and PDR in Kurdistan.

In the analysis sequence of *bla*<sub>veb-1</sub> gene from Iraqi bacterial isolate *P. aeruginosa* as depicted in Figure 3, the selected isolate is registered in the global gene bank. Locally *P. aeruginosa* isolates in Erbil city from clinical samples don’t match with global isolates and due to gene

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**Table 6.** Frequency of *bla*<sub>GES</sub> and *bla*<sub>veb-1</sub> gene in different clinical samples.

| Source         | Urine | Bronchial wash | Sputum | Wound | Burn | Total |
|----------------|-------|----------------|--------|-------|------|-------|
| *bla Ges* -5   | Positive | NO (%)     | 2(5%)  | 1(2.5%) | 2(5%) | 5(12.5%) | 6(15%) | 16(40%) |
|                | Negative | NO (%)     | 5(12.5%) | 3(7.5%) | 4(10%) | 4(10%) | 8(20%) | 24(60%) |
| *bla Veb -1*   | Positive | NO (%)     | 4(10%) | 1(2.5%) | 1(2.5%) | 5(12.5%) | 9(22.5%) | 20(50%) |
|                | Negative | NO (%)     | 3(7.5%) | 3(7.5%) | 5(12.5%) | 4(10%) | 5(12.5%) | 20(50%) |

**Table 7.** Frequency of *bla*<sub>veb-1</sub> gene in different patterns of multidrug resistance and different type of beta lactamase.

| Source         | Urine | Bronchial wash | Sputum | Wound | Burn | Total |
|----------------|-------|----------------|--------|-------|------|-------|
| ESBL           | NO (%) | 3(7.5%)     | 1(2.5%) | 3(7.5%) | 5(12.5%) | 8(20%) | 20(50%) |
| MBL            | NO (%) | 4(10%)      | 1(2.5%) | 2(5%) | 3(7.5%) | 7(17.5%) | 17(42.5%) |
| Biofilm        | NO (%) | 3(7.5%)     | 1(2.5%) | 2(5%) | 6(15%) | 7(17.5%) | 19(47.5%) |
| MDR            | NO (%) | 5(12.5%)    | 1(2.5%) | 2(5%) | 5(12.5%) | 7(17.5%) | 20(50%) |
| XDR            | NO (%) | 1(2.5%)     | 1(2.5%) | 1(2.5%) | 2(5%) | 4(10%) | 9(22.5%) |
| PDR            | NO (%) | 1(2.5%)     | 0       | 0      | 1(2.5%) | 2(5%) |       |

**Table 8.** Frequency of *bla*<sub>GES</sub> gene in different patterns of multidrug resistance and different type of beta-lactamase.

| Source         | Urine | Bronchial wash | Sputum | Wound | Burn | Total |
|----------------|-------|----------------|--------|-------|------|-------|
| ESBL           | NO (%) | 3(7.5%)     | 1(2.5%) | 3(7.5%) | 3(7.5%) | 6(15%) | 16(40%) |
| MBL            | NO (%) | 2(5%)      | 1(2.5%) | 2(5%) | 2(5%) | 6(15.5%) | 13(32.5%) |
| Biofilm        | NO (%) | 2(5%)     | 0(0%) | 2(5%) | 5(12.5%) | 7(17.5%) | 16(40%) |
| MDR            | NO (%) | 3(7.5%)    | 1(2.5%) | 2(5%) | 5(12.5%) | 5(12.5%) | 16(40%) |
| XDR            | NO (%) | 1(2.5%)     | 1(2.5%) | 1(2.5%) | 2(5%) | 4(10%) | 9(22.5%) |
| PDR            | NO (%) | 1(2.5%)     | 0       | 0      | 1(2.5%) | 2(5%) |       |
netic variation they may represent new isolates, thus the isolates were selected to register as a new isolate in the global gene bank. It is accepted under accession numbers (LOCUS MN900953) of nucleotides sequenced. The phylogenetic trees bla\textsubscript{VEB} gene encoding ESBL revealed a genotype closely related to others, registered in GenBank Table 9, from India, Iran, France, Brazil, Singapore, Kenya, USA, and China. Similar to the \textit{P. aeruginosa} gene, gene sequencing revealed a 99% similarity with other isolates deposited in GenBank.

Among the ESBL-producing isolates analyzed in the present study, 3 (8.5%) were carrying the \textit{bla\textsubscript{VEB}} gene and 32 (91.4%). The phylogenetic trees of the \textit{bla}\textsubscript{VEB} genes isolates were also recorded by Al-Ouqaili et al., 2020 (66), isolated from Al-Karkh General Hospital (Baghdad, Iraq) representing similarity to those isolated elsewhere (24 countries) including the USA, Poland, United Kingdom, France, Germany, South Korea, India, Kenya, and China., with compatibilities of 99%, and showed high similarity with similar isolates from other countries.

Genetic variation in the sequence of this gene which the isolates distributed in the phylogenetic tree. Following the comparison of the sequence of \textit{bla\textsubscript{VEB}}-1 in locally and global \textit{P. aeruginosa} isolates documented in NCBI, the phylogenetic tree showed similarity between the local and global isolates.

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None

**Interest conflict**

The authors declare no conflict of interest.

**Table 9.** BLAST results comparing the \textit{bla\textsubscript{VEB}}-1 gene from the \textit{P. aeruginosa} isolated in this study with those deposited in GenBank and its genetic distance from other global isolates.

| ACCESSION | country            | Source                             | Compatibility |
|-----------|--------------------|------------------------------------|---------------|
| ID: CP034908.2 | USA:Colorado | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: CP054591.1 | Nigeria: Ikeja | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: CP050328.1 | USA: Pennsylvania | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: CP050052.1 | USA | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: CP050054.1 | China | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: CP053706.1 | Taiwan | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: CP049161.1 | Australia: Queensland | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: CP047697.1 | China: Yangling | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: CP046069.1 | Belgium | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: CP045739.1 | Costa Rica | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: CP028959.1 | China: Daqing | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: LR590474.1 | United Kingdom | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: LR130528.1 | Switzerland | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: LS998783.1 | Germany | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: CP034429.1 | Russia: Moscow | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: CP030912.1 | South Korea: Seoul | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: CP029707.1 | Norway: Olso | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: CP016955.1 | Netherlands: Bilthoven | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: LN871187.1 | France | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: CP041787.1 | Kazakhstan: Almaty | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: CP038661.1 | India | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: CP023833.1 | France | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: CP035739.1 | Poland | \textit{Pseudomonas aeruginosa} | 99%           |
| ID: CP031449.2 | Ghana: Kumasi | \textit{Pseudomonas aeruginosa} | 99%           |
References

1. Shahcheraghi F, Nikbin VS, Feizabadi MM. Prevalence of ESBLs genes among multidrug-resistant isolates of pseudomonas aeruginosa isolated from patients in Tehran. Microb Drug Resist 2009;15:37–9.

2. Polotto M, Casella T, de Luca Oliveira MG, Rúbio FG, Nogueira ML, de Almeida MTG, et al. Detection of P. aeruginosa harboring bla CTX-M-2, bla GES-1 and bla GES-5,bla IMP-1 and bla SPM-1 causing infections in Brazilian tertiary-care hospital. BMC Infect Dis 2012;12:176.

3. Magiorakos AP. Multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) bacteria in healthcare settings. 2011.

4. Al-Kaisse AA, Al-Thwani AN, Al-Segar RQ. PCR detection of some ESBLs (bla genes in Pseudomonas aeruginosa isolated from burn’s units in Bagdad hospitals. J Biotechnol Res Cent 2015;9:74–80.

5. Dunne WM. Bacterial adhesion: Seen any good biofilms lately? Clin Microbiol Rev 2002;15:155–66.

6. Donlan RM, Costerton JW. Biofilms: Survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 2002;15:167–93.

7. Rao R, Karthika R, Singh S, Shashikala P, Kanungo R, Jayachandran S, et al. Correlation between biofilm production and multiple drug resistance in imipenem resistant clinical isolates of Acinetobacter baumannii. Indian J Med Microbiol 2008;26:333–7.

8. Oliver A, Mulet X, López-Causapé C, Juan C. The increasing threat of Pseudomonas aeruginosa high-risk isolates. Drug Resist Updat 2015;21–22:41–59.

9. Zafer MM, Al-Agamy MH, El-Mahallawy HA, Amin MA, Chandran S, et al. Evaluation of biofilm production by Pseudomonas aeruginosa isolates recovered from cystic fibrosis and non-cystic fibrosis patients. Brazilian J Microbiol 2012;38:1192–7.

10. Aktaś Z, Poirel L, Śalcioglu M, Ozcan PE, Midilli K, Bal Ç, et al. PER-1 and OXA-10-like β-lactamases in ceftazidime-resistant Pseudomonas aeruginosa isolates from intensive care unit patients in Istanbul, Turkey. Clin Microbiol Infect 2005;11:193–8.

11. Naas T, Poirel L, Karim A, Nordmann P. Molecular characterization of β-lactamase encoding genes among Pseudomonas aeruginosa strains isolated from cancer patients. Biomed Res Int 2014:2014.

12. Aktaš Z, Poirel L, Śalcioglu M, Ozcan PE, Midilli K, Bal Ç, et al. PER-1 and OXA-10-like β-lactamases in ceftazidime-resistant Pseudomonas aeruginosa isolates from intensive care unit patients in Istanbul, Turkey. Clin Microbiol Infect 2005;11:193–8.

13. Naas T, Poirel L, Karim A, Nordmann P. Molecular characterization of β-lactamase encoding genes among Pseudomonas aeruginosa strains isolated from cancer patients. Biomed Res Int 2014:2014.

14. Zhao WH, Hu ZQ. β-Lactamases identified in clinical isolates of Pseudomonas aeruginosa, Kuwait. Emerg Infect Dis 2001;7:468.

15. Zhao WH, Hu ZQ. β-Lactamases identified in clinical isolates of Pseudomonas aeruginosa, Kuwait. Emerg Infect Dis 2001;7:468.

16. Glupczynski Y, Bogaerts P, Deplano A, Berhin C, Huang TD, van Eldere J, et al. Detection and characterization of class a extended-spectrum-β-lactamase-producing Pseudomonas aeruginosa isolates in Belgian hospitals. J Antimicrob Chemother 2010;65:866–71.

17. CLSI. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-First Informational Supplement. Clin Lab Standards Inst - NCCLS 2011:31.

18. Eigner U, Schmid A, Wild U, Bertsch D, Fahr AM. Analysis of the comparative workflow and performance characteristics of the VITEK 2 and phoenix systems. J Clin Microbiol 2005;43:3829–34.

19. Yong D, Lee K, Yum JH, Shin HB, Rossolini GM, Chong Y. Imipenem-EDTA disk method for differentiation of metallo-β-lactamase-producing clinical isolates of Pseudomonas spp. and Acinetobacter spp. J Clin Microbiol 2002;40:3798–801.

20. Sharvari SA, Chitra PG. Evaluation of different detection methods of biofilm formation in clinical isolates of Staphylococci. Int J Pharma Bio Sci 2012;3:724–33.

21. Tamura K, Stecher G, Peterson D, Filipski A, Kumara S. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 2013;30:2725–9.

22. Kang CI, Kim SH, Kim H Bin, Park SW, Choe YJ, Oh MD, et al. Pseudomonas aeruginosa bacteremia: Risk factors for mortality and influence of delayed receipt of effective antimicrobial therapy on clinical outcome. Clin Infect Dis 2003;37:745–51.

23. Selina C. Pseudomonas infection Emeducine. World Med Libr 2002:1–10.

24. Fazul MKK, Zaini MZ, Rashid MA, Nazmul MHM. Antibiotic susceptibility profiles of clinical isolates of Pseudomonas aeruginosa from Selayang hospital, Malaysia. Biomed Res 2011;22:263–6.

25. Kamble DMDS. Phenotypic detection of ESBL and MBL in Gram Negative bacilli isolated from clinical specimens. Int J Med Res 2015;3:866–70.

26. Agrawal P, Ghosh A, Kumar S, Basu B, Kapila K. Prevalence of extended-spectrum-β-lactamases among Escherichia coli and Klebsiella pneumoniae isolates in a tertiary care hospital. Indian J Pathol Microbiol 2008;51:139–42.

27. Aky a, Salimi A, Nomanpour B, Ahmadi K. Prevalence and Clonal Dissemination of Metallo-Beta-Lactamase-Producing Pseudomonas aeruginosa in Kermanshah. Jundishapur J Microbiol 2015;8.

28. Al-Grawi IGA. Expression of mexAB-oprM Operon of Septicemic Pseudomonas aeruginosa in relation to antibiotic resistance 2011.

29. Gad GF, El-Domany RA, Zaki S, Ashour HM. Characterization of Pseudomonas aeruginosa isolated from clinical and environmental samples in Minia, Egypt: Prevalence, antibiogram and resistance mechanisms. J Antimicrob Chemother 2007;60:1010–7.

30. Chaudhary M, Payasi A. Rising antimicrobial resistance of Pseudomonas aeruginosa isolated from clinical specimens in India. J Proteomics Bioinforma 2013;6:5–9.

31. Wozniak DJ, Wyckoff TJO, Starkey M, Keyser R, Azadi P, O’Toole GA, et al. Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 Pseudomonas aeruginosa biofilms. Proc Natl Acad Sci U S A 2003;100:7907–12.

32. Jabalameli F, Mirsalehian A, Khoramian B, Aligholi M, Khosroo SS, Asadollahi P, et al. Evaluation of biofilm production in Pseudomonas aeruginosa biofilms. Proc Natl Acad Sci U S A 2015;12:3227–32.

33. O’Toole GA, et al. Int J Curr Microbiol Appl Sci 2016;5:269–74.

34. Emami S, Eftekhar F. The Correlation Between Biofilm Formation and Drug Resistance in Nosocomial Isolates of Acinetobacter baumannii. Avicenna J Clin Microbiol Infect 2015;2:23954–23954.

35. Lyra P, Cugati S, Anuradha K, Venkatesha D. Metallo beta lactamase-producing clinical isolates of Pseudomonas aeruginosa isolated from patients in Tehran. Microb Drug Resist 2009;15:3798–801.

36. Punia P, Goel N, Asati S, Phogat R, Chaudhary U. Biofilm detection amongst Extended Spectrum Beta Lactamase ( ESBL ) and Metallo Beta Lactamase ( MBL ) producing clinical isolates of Acinetobacter baumannii. Int J Enchanced Reasearch Med Dent Care
H, Kalantari N. Detection of VEB-1, OXA-10 and PER-1 genotypes in Pseudomonas aeruginosa strains isolated from burn patients. Burns 2010;36:70–4.

Amirkamali S, Naserpour-Farivar T, Azarhoosh K, Peymani A. Distribution of the blaOXA, blaVEB-1, and blaGES-1 genes and resistance patterns of ESBL-producing Pseudomonas aeruginosa isolated from hospitals in Tehran and Qazvin, Iran. Rev Soc Bras Med Trop 2017;50:315–20.

de Almeida Silva K de CF, Calomino MA, Deutsch G, de Castilho SR, de Paula GR, Estep LMR, et al. Molecular characterization of multidrug-resistant (MDR) Pseudomonas aeruginosa isolated in a burn center. Burns 2017;43:137–43.

Tawfik AF, Shbil AM, Aljohi MA, Altammani MA, Al-Agamy MH. Distribution of Ambler class A, B and D β-lactamases among Pseudomonas aeruginosa isolates. Burns 2012;38:555–60.

Pica R, Poire L, Gales A, Nordmann P. Diversity of -Lactamases Produced by Cefazidime-Resistant Pseudomonas aeruginosa Isolates Causing bloodstream Infections. Brazil Antimicrob Agents Chemother Am Soc Microbiol 2009;53:3908–13.

Khoshnood S, Khosravi A, Jomehzadeh N, Montazeri E, Motahar M, Shali F, et al. Distribution of extended-spectrum β-lactamase genes in antibiotic-resistant strains of Pseudomonas aeruginosa obtained from burn patients in Ahvaz, Iran. J Acute Dis 2019;9:53–7.

Poirel L, Le Thomas I, Naas T, Karim A, Nordmann P. Biochemical sequence analyses of GES-1, a novel class A extended-spectrum β-lactamase, and the class 1 integron In52 from Klebsiella pneumoniae. Antimicrob Agents Chemother 2000;44:622–32.

Paterson DL, Bonomo RA. Clinical Update Extended-Spectrum Beta-Lactamases: a Clinical Update. Clin Microbiol Rev 2005;18:657–86.

Ahmed OB, Asghar AH, Bahwerth FS. Prevalence of EsbI Genes of Pseudomonas Aeruginosa Strains Isolated From Makkah Hospitals, Saudi Arabia. Eur J Biol Med Sci Res 2015;3:12–8.

Rios P, Rocha C, Castro W, Vidal M, Canal E, Bernal M, et al. Extensively drug-resistant (XDR) Pseudomonas aeruginosa identified in Lima, Peru co-expressing a VIM-2 metallo-β-lactamase, OXA-1 β-lactamase and GES-1 extended-spectrum β-lactamase. JMM Case Rep 2018;5.

Azzez, S., Jafar, S., Aziziaram, Z., Fang, L., Mawlood, A., Ercisi, M. Insulin-producing cells from bone marrow stem cells versus injectable insulin for the treatment of rats with type I diabetes. Cell Mol Biomed Rep 2021;1(1): 42-51.

Bilal I, Xie S, Elburki M, Aziziaram, Z, Ahmed S, Jalal Balaky C. Cytotoxic effect of diferylulymethane, a derivative of turmeric on different human glioblastoma cell lines. Cell Mol Biomed Rep 2021;1(1): 14-22.

Kazemi E, Kahrizi D, Moradi MT, Sohrabi M, Amini S, Mousavi SA, Yari K. Association between Helicobacter pylori hopQI genotypes and human gastric cancer risk. Cell Mol Biol 2016;62(1):6-9.

Kazemi E, Kahrizi D. Lack of association between gastrointestinal and hopQ alleles in Helicobacter pylori. Genetika. 2016;48(3):893-902.

Bren B, Zhang J, Ma Q. The relationship between the simultaneity present of cagA and hopQI genes in Helicobacter pylori and the risk of gastric cancer. Cell Mol Biol 2021;67(2):121-6.

Al-Ouqaili MT, Khalaf EA, Al-Kubaisy SH. DNA Sequence Analysis of BlaVEB Gene Encoding Multi-drug Resistant and Extended-spectrum β-lactamases Producer Isolates of and. Open Microbiol J 2020;14(1).