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Prevalence of Metallo β-Lactamase Genes Among MDR *Pseudomonas Aeruginosa* Isolated from Different Sources in Baghdad

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ABSTRACT: ***Background:*** *Pseudomonas aeruginosa is a gram negative apportunistic pathogen that has ability to couse different type of infections and charecterised by their high resistance to commonly used antibiotics via different stratiges.* ***Objective:*** *The objective of this study is to detect the prevalence rate of MβL gene among MDR Pseudomonas aeruginosa.* ***Methods:*** *Forty clinical isolates of Pseudomonas aeruginosa were obtained from different hosptials in Baghdad/Iraq. Their identification was confirmed by using 16s rDNA as a housekeeping gene (reference gene). Specific primers were used to detect 3 types of Metallo β-lactamase (MβL) genes vim, spm1, and imp1 genes followed by sequencing the amplified fragment which was analyzed by Geneious software. Antibiotic sensitivity test for 15 antimicrobial agents was done using the Kirby-Bauer disc diffusion method.* ***Results:*** *The revealed resistance pattern was as following: 100% for the combination of Trimethoprim/Sulphamethoxazole and Nitrofurantoin, 95% for Tigecycline, 82.5% for Ciprofloxacin, 67.5% for Cefepime, 60% for Levofloxacin, 57.5% for Carbenicillin, 55% for Piperacillin, 50% for Amikacin, 47.5% for Tobramycin, 45% for both Imipenem and Ceftazidime, 27.5% for piperacillin/tazobactam, 22.5% for Aztreonam and 7.5% for Colistin. More than half (55%) of isolates were positive for MβL enzymes production during phenotypically assessed by combined disc test. The MIC of meropenem ranged from 16μg/ml to 32 μg/ml. The percentage of MβL genes among the total isolates during conventional PCR was as follows: 95% for vim, 25% of the isolates harbored both vim and imp1, while spm 1 are negative in all isolates. The result of the study explained that 10 isolates from the antibiotic susceptibility test were resistant to 10-14 antimicrobial agents that harbored both vim and imp1 genes.* ***Conclusions:*** *MβL gene could be used as a genetic marker to determine the degree of resistance in MDR P. aeruginosa which are more resistant than other isolates that have one gene of MβL responsible for break the β-lactam ring then inactivating the β-lactam antibiotic.*

KEYWORDS: *MβLs; MDR P. aeruginosa; Pairwise identity; Sequencing; 16s rDNA housekeeping*

INTRODUCTION

Pseudomonas aeruginosa is major opportunistic pathogen being one of the most frequent causes of acute infection in immunocompromised persons and hospitalized patients [1]. In addition, it is considered as a major nosocomial pathogen because it has a huge rate of emergence, acquisition, and spread of different resistance mechanisms including intrinsic, acquired, and adaptive resistance [2]. On the head of these resistance mechanisms are β-lactamases, which are definable as a family of enzymes that vary in their spectrum of activity against the β-lactams group and are considered as the primary cause of bacterial resistance against them [3]. The mechanisms of action of these enzymes are represented by cleaving the amide bond in the four-atom β-lactam ring structure in

β-lactam, hence rendering them nonfunctional [4]. They are produced as extracellular enzymes by Gram-positive bacteria while remaining within the periplasmic space in Gram-negative ones [5]. The gene encoding for the β-lactamase enzyme is either mediated on a bacterial chromosome or present on mobile genetic elements such as plasmids, transposons, and integrons [6]. They are classified into four molecular classes (A, B, C, and D) based on their amino acids sequence similarity, and this classification is known as the Ambler classification [7]. As well as, β-lactamase enzymes are classified into groups 1, 2, and 3 according to β-lactam substrates and the inhibitors that inactivate enzyme activity, this classification is known as the Buch-Jacoby-Medeiros classification [8]. Class B of Ambler classification is known as metallo β-lactamase because they have zinc ions in the active site , which is required for the catalytic activity of the β-lactam ring [9]. *MβLs* belong to Buch-Jacoby functional group 3 and they have a broad substrate spectrum that catalyzes.

The hydrolysis of all β-lactams with the exception of monobactams [10]. Their enzymic activity is suppressed by chelating agents such as ethylenediaminetetraacetic acid (EDTA) [11]. The B1 subclass enzymes were the first *MβL* described to need two Zn (II) ions to be fully activated [12]. The most important clinically and largest number of *MβLs* have been identified belong to the B1subclass [13]. The gene encoding for most of these B1 subclass enzymes was disseminated worldwide on mobile genetic elements aggravating the geographical spread of resistance [14]. Multiple variants of these enzymes are found in clinical isolates of Gram-negative resistant pathogens such as *P. aeruginosa* [15]. This study aimed to study the correlation between the availability and prevalence of *MβLs* and MDR among local *P. aeruginosa* isolates.

MATERIALS AND METHODS

Forty clinical isolates of *P. aeruginosa* were obtained from different infections including 11 isolates from sputum, 7 isolates from the wound, 7 isolates from urine, 6 isolates from the ear, 4 isolates from burn, 3 isolates from blood, and only 2 isolates from cerebrospinal fluid. All these isolates were collected from different hospital clinical laboratories in Baghdad.

Morphological Identification

The *P. aeruginosa* isolates were identified on selective medium (*Pseudomonas* cetrimide agar) depending on morphological characteristics involved in their color, colony, shape, and pigment production [16].

DNA Extraction and Molecular Study

According to the protocol of the ABIO pure extraction kit, the genomic DNA was isolated from bacterial growth of all forty clinical isolates, and *16s* rDNA was used as a housekeeping gene (HKG) for molecular identification of *Pseudomonas* isolates [17]. The product size and primers sequence of HKG, *vim, spm1, and imp1* are listed in Table 1. The forward and reverse primers were prepared according to information from manufacturing companies. The PCR mixture of the mentioned gene (HKG, *vim, spm1, and imp1*) was composed of 12.5 µL of Go Taq® Green Master Mix (Promega company) 2 µL template DNA, 1 µL for each of forward and reverse primers (final concentration was 0.4 pmol/µL), and 8.5 µL deionized nuclease-free water to reach the final volume 25 µL. All these components of the PCR mixture were put in the Eppendorf tubes and then mixed shortly with vortex before being placed in the PCR machaine and then submitted to the following conditions of the conventional PCR run: 2 minutes of the initial denaturation step at 95°C followed by 30 repeated cycles, Each cycle consists of three steps, the first step denaturation at 94 °C for 30 Sec, the second step annealing at 58 °C for 60 Sec and the third step extension for 30 sec at 72 °C followed by final extension step which it was at 72 °C and for 5 min. The *MβL* genes were detected by using specific primers listed in Table 1, and the final optimizing condition in Table 2 was fixed after gradient PCR was performed in the current study. After the end of conventional PCR amplification , the amplified genes (products) were subjected to electrophoresis.

Gel Electrophoresis

The agarose powder (1%) is mixed with an electrophoresis TBE (1x) buffer and heated to a high temperature until all of the agarose powder was melted, then poured into a gel casting tray, and a comb was placed at one end to make wells for the samples to pipetted and removed it after the gel was cooled finally, the gel was stained with fluorescent dye that binds with DNA five microliters from

the product were loaded to wells on the gel then exposing it to an electric field (100 vol/45 min) and the result was visualized under UV-transilluminator apparatus as bright bands due to the fluroresent dye ,while the other (20 µL) from the amplified genes during conventional PCR that was stored at -20 °C and sequenced by Macrogen company, Korea later analyzed through the usage of Geneious software (version 2019 prime). Results were read by comparing them with the NCBI control strains. Query, pairwise alignment, and identity; were also anatomized with the same software.

Table 1. Primers used in this study

Primer name	Oligo Sequence 5'3'	Product size (bp)	Reference
16s rDNA	F AGAGTTTGATCCTGGCTCAG R CTTGTGCGGGCCCCCGTCAATTC	956	[18]
Vim	F GGTCTCATTGTCCGTGATGGTG R GGAATCTCGCTCCCCTCTACCT	242	[19]
imp1	F ACCGCAGCAGAGTCTTTGCC R ACAACCAGTTTTGCCTTACC	587	[20]
spm1	F GCGTTTTGTTTGTTGCTC R TTGGGGATGTGAGACTAC	786	[20]

Primers were synthesized by Macrogen Humanizing Genomics /South Korean)

Table 2. PCR programs used for amplification *MβL* genes in *P. aeruginosa* isolates

Amplified gene	Initial denaturation	Cycle No.	Denaturation	Annealing	Elongation	Final extension
16srDNA	95 °C/5 min	30	94 °C/30 sec	58 °C/1 min	72 °C/30 sec	72 °C/5 min
vim	94 °C/5 min	40	94 °C/45 sec	57.5 °C/45 sec	72 °C/45 sec	72 °C/5 min
imp1	96 °C/10 min	35	96 °C/1 min	56.2 °C/30 sec	72 °C/1 min	72 °C/10 min
Spm	96 °C/10 min	35	96 °C/1 min	56.2 °C/30 sec	72 °C/1 min	72 °C/10 min

Antibiotic Susceptibility Test and MIC for *P. aeruginosa* Isolates

The isolates which identified as *P. aeruginosa* were submitted to antibiotic susceptibility test by Kirby-Bauer method toward different fifteen antimicrobial agents and were interpreted according to clinical and laboratory standard institute (CLSI 2022) for *P. aeruginosa* [21]. The antibiotics were used; Carbenicillin CAR (100 µg), Piperacillin PRL (100 µg), Ceftazidime CAZ (30 µg), Cefepime FEP(10 µg), Imipenem IPM (10µg), Aztreonam ATM (30 µg), piperacillin /tazobactam PIT (100/10 µg), Colistin COL (10 µg), Amikacin AK (10 µg), Tobramycin TOB (10 µg), Ciprofloxacin CIP (5 µg), Levofloxacin LEV (5 µg), Trimethoprime/Sulphamethoxazole SXT (1.25/23.75 µg), Tigecycline TGC (15 µg), Nitrofurantoin F (100 µg). Furthermore, the MIC of meropenem for more resistant isolates was determined by serial dilution technique and the concentration of bacterial cell was adjusted to 1.5×10^8 CFU/ml. A 100 µl from bacterial suspension was transferred to 5ml tube of Mueller-Hinton broth. After an incubation period at 37 °C during 18-24 h, resazurin dye 500 µl was added to tubes that contain the bacterial suspension and meropenem dilution (from 2 µg to 32 µg). The MIC results were read by distinguishing the color alteration. The pink and red colors indicate bacterial growth while the blue color indicates no growth.

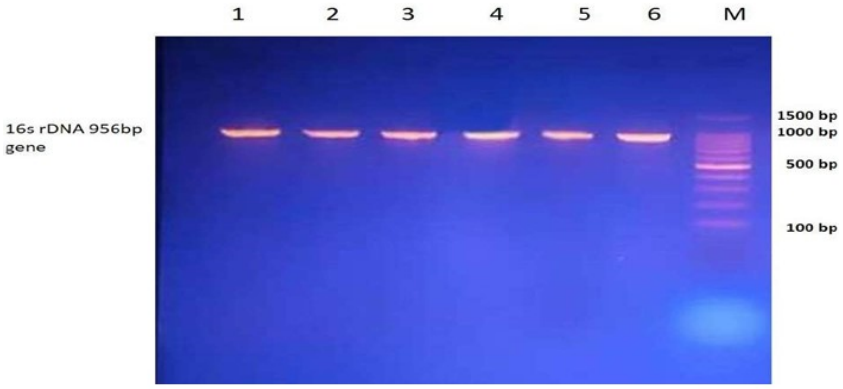
Phenotypic detection of *MβLs*

All of the identified *P. aeruginosa* in the current study were submitted to a combined disc test (CDT) to detect the MBL production in *P. aeruginosa* phenotypically. The concentration of bacterial cells was adjusted to 1.5×10^8 CFU/ml compared to 0.5 Mcfarland and was spread on Mueller-Hinton agar (MHA) plates. Two imipenem discs were placed on inoculated plates and 5 µl of EDTA solution was added to one of the discs. After overnight incubation, if the inhibition zone around IMP+EDTA discs was increased by about $\geq$7 mm compared to IMP disc alone then considered as *MβL* producer isolate [22].

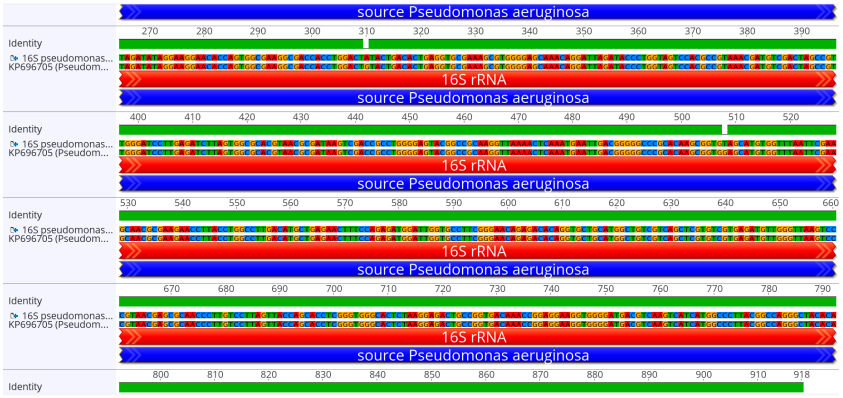
RESULTS AND DISCUSSION

Collection and identification of bacterial isolates

The forty isolates in the current study were collected from different sources (sputum, wound, urine, ear, blood, burn, and CSF because *P. aeruginosa* is a more significant human pathogen, responsible for the high rate of nosocomial infection which reached 10-15% and it has a huge rate of resistance mechanisms toward a wide range of antimicrobial agents [23]. Colonies of *P. aeruginosa* isolates appeared highly distinguised on the *Pseudomonas* cetrimide agar which it was considered a selective and differential medium for the identification of the *P. aeruginosa* because it contains cetrimide that acts as an inhibitor of most bacteria and enhances the production of the two special pigments pyocyanin and pyoverdin that responsible for recognition of distinctive colonies of *P. aeruginosa* [24]. For the genotypic identification of *P. aeruginosa* isolates, all forty isolates in the current study showed a PCR product for *16s* in conventional PCR technique as a molecular method for identification by using *16s* rDNA as housekeeping gene (HKG). Huge diversity of species in this genus share the same morphological and cultural characteristics, The traditional laboratory techniques are time-consuming, as well as similar results of biochemical tests. Thus, all these reasons lead to depending on this molecular method to confirm the identification [25]. The identification of *Pseudomonas* based on *16s* rDNA sequence represents rapid and accurate PCR assays that allow the differentiation of *P. aeruginosa* from other *Pseudomonas* species [17]. Figure 1(a) illustrates the result that shows the size of amplicon 956 bp which matched the size of *16s* rDNA (HKG). Figure 1(b) illustrates the pairwise identity (99.89%) which exemplifies residues percentage that were identical in alignment with gaps versus non-gap residues. Few differences appeared between the local isolate and NCBI standard strain FM 25 *16s* ribosomal RNA gene, partial sequence. Accession KP696705 version KP696705.1 db_xref="taxon:287"



(a)

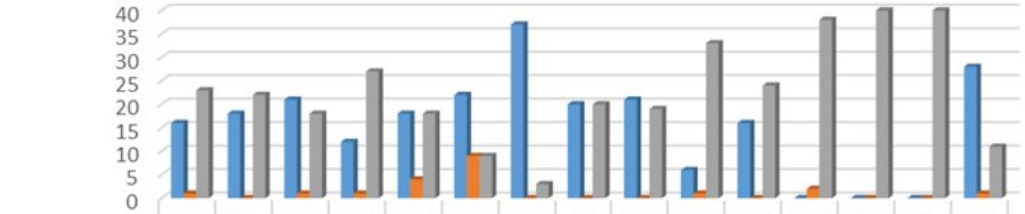


(b)

Figure 1. (a) Agarose gel electrophoresis (100 vol/45min) for *16s* rDNA gene (amplified size of 956bp), as compared with DNA ladder (M), from 1 to 6 lanes represent the positive isolates of *P. aeruginosa* for *16s* rDNA gene. (b) Alignment of *16s* rDNA gene with the same gene of NCBI standard strains *P. aeruginosa* (MH114980) , sequence data were analysed using geneious prime software version 2019

Phenotypic Detection of Antibiotic Resistance Pattern and MIC Determination

The result of the antibiotic susceptibility test in Figure 2 displayed various patterns of resistance associated with several groups of tested antimicrobial agents. *P. aeruginosa* isolates in the current study showed the high rate of resistance toward nitrofurantoin and trimethoprim/sulfamethoxazole reached 100% followed by 95% for tigecycline, while the ratio (7.5%) represents the high rate of sensitivity toward colistin on the other hand, *P. aeruginosa* isolates showed a range of resistance between 22.5 – 80% for aztreonam, ceftazidime, imipenem, piperacillin, carbenicillin, tobramycin, amikacin, cefepime, levofloxacin, and ciprofloxacin in a dramatic increasing pattern . The result was agreed with the researcher study in Germany [26], who reported the resistance rate of the following antimicrobial agents: Colistin, piperacillin /tazobactam, Ceftazidime, Imipenem, Piperacillin, Tobramycin, Cefepime, Levofloxacin, and Ciprofloxacin. The resistance rate of Aztreonam and Amikacin was agreed with the researcher's study in Romania [27]. The resistance rate of Carbenicillin was agreed with the researcher's study in India [28]. The rate of resistance against Tigecycline was agreed with the study in Riyadh/Kingdom of Saudi Arabia [29]. The nitrofurantoin resistance rate was agreed with the researcher's study in Turkey [30]. The resistance against combination trimethoprim/sulfamethoxazole was agreed with the researcher's study in Egypt [31]. The MIC step of meropenem was used as a proactive step (induction) to find out which of the isolates had a high production of *MβL* and any antibiotic from the carbapenem group expressed the same content in terms of the calculated breakpoint in which the isolates were characterized as resistant if the MIC was greater than breakpoint , while considered as susceptible if the MIC was lower than breakpoint [21]. The MIC of meropenem for more resistant ten isolates in AST ranged from 16 µg/ml in five isolates to 32 µg/ml in the other five and this result was agreed with the study in the USA [32], who submitted that the MIC of meropenem was ranged from 16-32 µg/ml.



	CAR	PRL	CAZ	FEP	IMP	ATM	COL	AK	TOB	CIP	LEV	TGC	SXT	F	PIT
Sensitive	16	18	21	12	18	22	37	20	21	6	16	0	0	0	28
Intermediate	1	0	1	1	4	9	0	0	0	1	0	2	0	0	1
Resistant	23	22	18	27	18	9	3	20	19	33	24	38	40	40	11

Figure 2. The percentage resistance of *P. aeruginosa* isolates ,Carbenicillin(CAR), Piperacillin(PRL), Ceftazidime(CAZ), Cefepime(FEP), Imipenem(IPM), Aztreonam(ATM), piperacillin /tazobactam(PIT), Colistin(COL), Amikacin(AK), Tobramycin(TOB), Ciprofloxacin(CIP) , Levofloxacin(LEV), Trimethoprime/Sulphamethoxazole(SXT), Tigecycline (TGC), Nitrofurantoin(F)

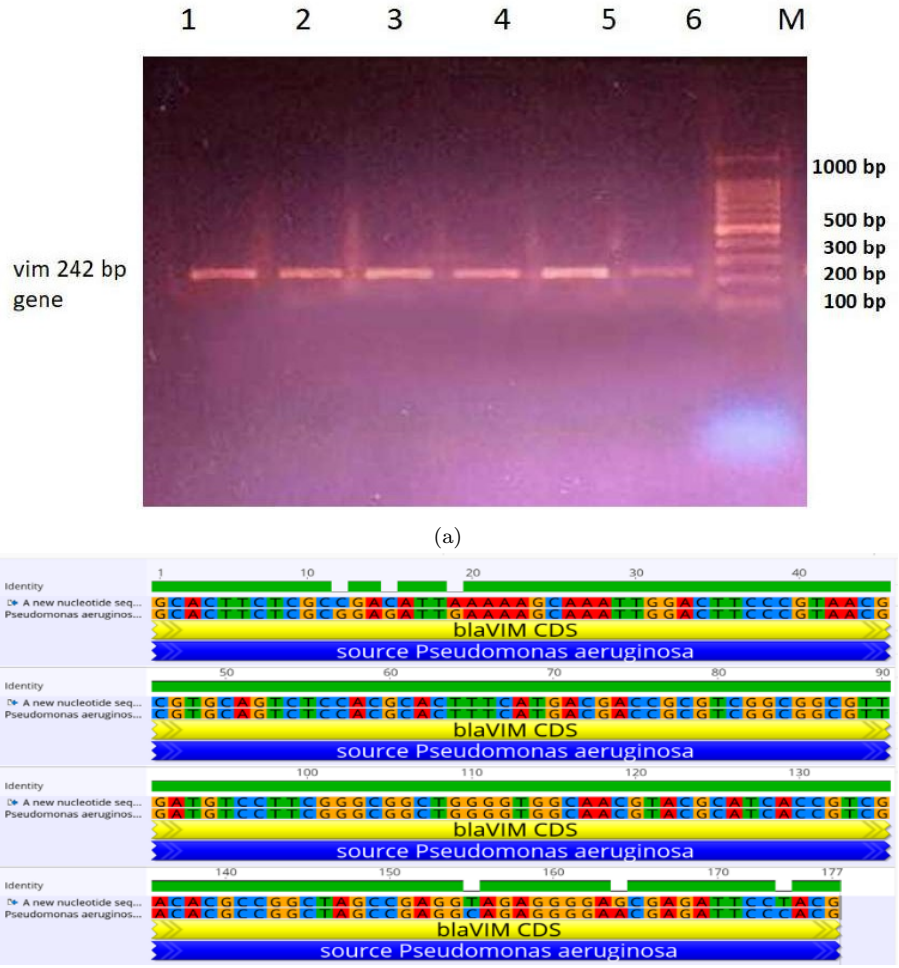
Phenotypic Detection of *MβLs* of *P. aeruginosa* Isolates

The results showed that 22/40 (55%) of *P. aeruginosa* isolates were positive for metallo β-lactamase production during a combined disc test by using EDTA (EDTA act as inhibitor to *MβL* due to binding with zinc ion in active site) this result was agreed with a researcher study done in Tehran /Iran [33], who reported that the *MβL* production in *P. aeruginosa* isolates was 68.6%, while in another study done in Bathinda/India submitted that the positive results for *MβL* production by *P. aeruginosa* reached to 21.8% [34], which was lowest than current study results. These variations in results might be due to differences in antibiotic usage and intelligent selection of antibiotics in their hospitals [14].

Genotypic Detection of MBL Gene

The *vim* and *imp1* genes belong to the B1 subclass of *MβL* and they have a broad-spectrum substrate profile against penicillins, cephalosporins, and carbapenems [12]. Investigation of the *vim* gene revealed positive results and the percentage was 38/40 (95%) for *P. aeruginosa* isolates. An obvious band on agarose gel appeared and the expected size of the amplicon was 242bp in the distance middle between 200 and 300bp of DNA ladder bands which match the size of the specific *vim* primer Figure 3(a). This result was agreed with a researcher in Korea [35] who illustrated that the prevalence

rate of the *vim* gene among *P. aeruginosa* isolates was 100%. Another study in Brazil [36], reported that the prevalence rate of the *vim* gene among *P. aeruginosa* isolates was 30.6% and this result does not agree with the current study result. Sequencing results revealed that the identity with local isolate was 97.8% Figure 3 (b).



(a)

(b)

Figure 3. (a) Agarose gel electrophoresis (1%agarose,50vol/60min) for *vim* gene (amplified size 242bp), as compared with DNA ladder(M), from 1 to 6 represent the positive isolates of *P. aeruginosa* for *vim* gene .(b) Pairwise identity and nucleotide sequence for *vim* gene , sequence data were analysed using geneious prime software version 2019

Amplification of the *imp1* gene in the current study showed that 10/40 (25%) of *P. aeruginosa* isolates harbored this gene with probable size 587bp amplicon, Figure 4(A). The result of the current study agreed with the researcher's study done in China [37], who submitted that the prevalence rate of *imp1* gene among *P. aeruginosa* isolates was 28.2%, while in another study done in Brazil [36], who showed that the prevalence rate of *imp1* gene among *P. aeruginosa* isolates as 8.3% which was lower than current study result. Pairwise identity represented 99.8% of Figure 4(b), while the *spm1* was not observed in all 40 isolates of the current study.

The result explained above demonstrated that the isolates of *P. aeruginosa* (ten) which possess both of *MβL* genes (*vim* and *imp1*) were resistant to 10 - 14 antimicrobial agents in AST which more resistant than isolates were harbored one of *MβL* genes.

The treatment of *P. aeruginosa* infections continues to be a significant challenge because the huge capacity of *P. aeruginosa* to develop or acquire new resistance mechanisms to new antibiotics then contributes to the development of multidrug-resistant strains, and makes conventional antibiotics ineffective for the treatment of infections so that the overuse and misuse of antibiotics pose serious concerns for public health. The non-antibiotic therapeutic approaches, especially quorum sensing inhibition, phage therapy and the use of nanoparticles, have shown significant antimicrobial effects

against antibiotic-resistant strains of *P. aeruginosa* in vitro or in animal models, and they are being considered as alternatives or adjuncts to conventional antibiotics.

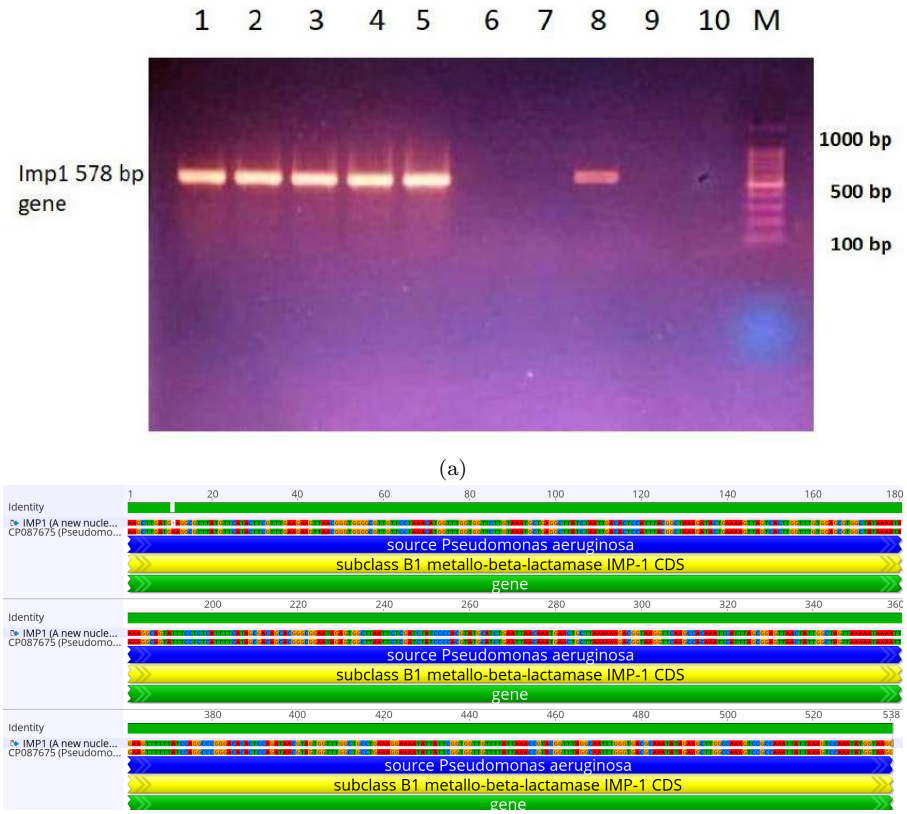


Figure 4. (a) Agarose gel electrophoresis (1% agarose, 50vol/60min) for *imp1* gene (amplified size 587bp), as compared with DNA ladder (M), Lanes 1 ,2 ,3 ,4 ,5 ,8 were positive results with 587bp *imp1* and lanes 6 ,7 ,9,10 showed negative results. (b) Pairwise identity and nucleotide sequence for *imp1*, sequence data were analysed using geneious prime software version 2019

CONCLUSION

The high prevalence rate of the *MβL* genes was among the forty *Pseudomonas aeruginosa* isolates in the current study as well as high levels of intrinsic or acquired resistance mechanisms make these isolates multidrug resistance or extensive drug resistance against more effective antimicrobial agents such as *β*-lactam, aminoglycoside and quinolones group. On the other hand, there are direct proportion between the resistance of the isolates to these antimicrobial agents with the presence of *MβL* genes and their numbers, while DNA sequencing is a very important method to confirm the identification and determine if the gene of region that regulates the gene contains changes (variants) or mutations.

SUPPLEMENTARY MATERIAL

None.

AUTHOR CONTRIBUTIONS

Mustafa Azm Saidmurad: Visualization, writing, and editing. Sawsan Sajid Al-Jubori: Suggested the idea, and analysed genomic data. Mohamed Faraj Edbeib: Investigation, and review of the final.

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None.

DATA AVAILABILITY STATEMENT

None.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ETHICAL APPROVAL

The Ethics Committee of Mustansiriyah University, College of Science reviewed and proved this study under the ethical approval reference number BCSMU/1221/0009M.

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