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Molecular Detection of Multidrug Resistant of Some Genes and the Effect of ZnONPs as Alternative to Antibiotics for *Pseudomonas aeruginosa*

A Thesis
Submitted to the Council of the College of Education for Pure Science, University of Diyala, in Partial Fulfillment of the Requirements for the Degree of Doctorate of Philosophy in Biology

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

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We certify that this thesis (**Molecular Detection of Multidrug Resistant of Some Genes and the Effect of ZnONPs as Alternative to Antibiotics for *Pseudomonas aeruginosa*)** was prepared by **Lina Abdulameer Salman**, under our supervision at the Department of Biology, College of Education for Pure Science, University of Diyala, Iraq. As partial fulfillment of the requirements for the degree of doctor philosophy in Biology.

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Dedication

To the great creator ... The God

To those who have given their lives to our Iraq

To the strong wedge and the towering mountain who guided me with love … My father

To the love, kindness, and the candle of my life … My Mother

To my soul .. my partner and support… My Husband Saif

To my little angels ……My lovey children Mohammed .. Yousif … Rahaf

To the pure hearts and loyal hands who assisted me in life …My Brothers and Sister

To all who supported me

Lina
2019

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Yours Truly

Lina Abdulameer

Summary

A total of (326) clinical samples were collected from burns, wounds, sputum, urine and otitis media infections from patients who attended different hospitals in Diyala province during the period from February to August /2018. The results showed that bacterial isolates appeared in 293of these samples. The conventional microbiological methods, VITEK 2 automated system and genetic detection by 16S rRNA showed that 81(27.6%) of the isolates were *Pseudomonas aeruginosa* isolates.

The resistance and sensitivity of *P. aeruginosa* isolates to (18) antibiotics from different classes were verified by Kirby-Bauer standard disk diffusion method to evaluate their resistance. There was significant increase ($p<0.01$) in the resistance rate of *P. aeruginosa* to different antibiotics. It was (93.82%) to Amoxicillin-Clavulanic acid, (90.12%) to Streptomycin, (87.65%) to Ceftriaxone, (85.18)% to Ticarcillin, (85.18%) to Cefotaxime, (85.18%) to Gentamicin, (80.24%) to Cefepime, (75.30%) to Ceftazidime, (74.07) to Piperacillin, (72.83%) to Levofloxacin and (71.60%) to Ticarcillin/Clavulanic acid when compared with its resistance rate to Oflaxacin, Ciprofloxacin, Tobramicin, Amikacin and Aztreonam which were (69.13%), (67.90%), (65.43%), (56.79%) and (50.61%) respectively, while there was a significant decrease in its resistance rate to Meropenem (23.45%) and Imipenem (11.11%). In this investigation, antibiotic susceptibility testing of *P. aeruginosa* isolates showed that 20(24.69%), 25(30.8%), 27(33.33%) and 9(11.11%) of the isolates were multi drug sensitive (MDS), multi drug resistant MDR, extensively drug resistant (XDR) and pan drug resistant (PDR) respectively. Based on the results of the susceptibility testing, 19 (23.45%) of *P. aeruginosa* isolates were found to be resistant to at least one of the Carbapenems. Resistance to Carbapenems by disk diffusion was shown in 9(11.11%) isolates for both Meropenem and Imipenem, and in 10(12.34%) isolates for Meropenem alone. In current study, (19) isolates of Carbapenem resistant *P. aeruginosa* were selected to perform the screening analysis of β-lactamase genes, aminoglycoside resistant genes and efflux pump genes using the PCR technique.

Two-fold dilution method was used to determine the minimum inhibitory concentrations (MICs) and minimum bactericidal concentration (MBC) for three antibiotics, and the results showed that there were differences in MIC and MBC values. All the 19(100%) isolates were able to grow in high concentrations of Gentamicin MIC ranged from (64–1024 µg/ml), MBC ranged from (256- >1024 µg /ml), MIC of Ceftazidime ranged (64-512 µg /ml) and MBC was (128- >1024). Imipenem was the most effective antibiotic as MIC ranged from (2–256 µg /ml) and MBC ranged from (4- 512 µg /ml). MIC of ZnO NPs ranged (325-5200 µg/ml) on Carbapenem resistant *P. aeruginosa* isolates.

ESBLs genes ($bla_{\text{OXA-10}}$ and bla_{PER}) were screened by PCR technique for the isolates. The results of gel electrophoresis for PCR product by using specific primers for these genes showed that 12(63.15%) of the isolates were positive for $bla_{\text{OXA-10}}$ gene. However, none of the (19) Carbapenem-resistant isolates of *P.aeruginosa* had the bla_{PER} ESBL gene. Out of 19 of Carbapenem resistant *P. aeruginosa* isolates,16 (84.21%) were found to produce MβL. Among the (16) phenotypic Metallo β-lactamase isolates the results of PCR revealed that 9(56.25%) isolates had bla_{VIM} genes, while 4(25%) isolates carried bla_{NDM} genes, and no bla_{IMP} was detected among Carbapenem resistant strains in this study.

In the current study, three (3) genes encoding the aminoglycoside modifying enzymes (AME) were detected. These genes were *aac(6')-Ib, aac(3')-II and ant(4')-IIb.* Results of Uniplex PCR method showed that the *aac(6')-Ib* was the most prevalent AME gene since it was found in 18/19 (94.73%) of the isolates, followed by *ant(4')-IIb* and *aac(3')-II* whose rates were 10.52% (2/19) and 5.26%(1/19) respectively.

Based on the results of Uniplex PCR,it was found that 19(100%) of Carbapenem resistant *P.aeruginosa* isolates gave positive results for efflux system *MexY* gene, and based on the results of Multiplex PCR, 18(94.7 %) isolates had *MexB* and *MexF* genes, while 17(89.47%) isolates had *MexD* gene. This may indicate the prevalence of these types of resistance in the current isolated bacteria. The results revealed that

18/19 (94.7%) of the isolates were harboring both AMEs and efflux genes. It is noticed that most of the isolates were harboring more than one of the efflux pump genes in corporation with one or more of AME genes. It was also shown that 12/19(63.15%) of the isolates were harboring both ESβLs and efflux genes.

Depending on the detection of Integrase gene for the investigation of class 1 integron, PCR assay showed that 19(100%) of the Carbapenem resistant *P. aeruginosa* isolates were integrase gene positive, which confirms the extremely high dissemination of class 1 integron at the hospitals of Baquba in Diyala province. Analysis of class 1 integron variable regions showed the presence of (4) different fragment sizes of approximately 300, 400,600 and 1500 bp. Current results also showed that 9(47.36%) isolates carried class 1 integron with sizes of approximately 1.5 kb, 2(10.52%) isolates with sizes of approximately 600bp and 400 bp as well as 3(15.78%) isolates with sizes of approximately 300bp. However, the analysis of the integron variable region revealed that 3/19(15.78%) isolates were lacking the gene cassettes, indicating a low occurrence of empty class 1 integrons among these strains.

Enterobacterial repetitive intergenic consensus amplification of (19) clinical Carbapenem resistant *P.aeruginosa* isolates showed that 19(100%) of them revealed at least one amplification band. ERIC-PCR typing showed two group **A and B** with **6** unique isolates, showing similar antimicrobial resistance patterns within the same group.

From human sources (2) strains of Carbapenem resistant *P. aeruginosa* were isolated in Baquba city/Diyala and each sequence had a symbol code (SL221814, SL8283) 16S rRNA gene sequences submitted to the Gen Bank. This strain was published in the national center for biotechnology information (NCBI), while the database of the strain was recorded in the DNA Data Bank of Japan (DDBJ) and Gene Bank for DNA sequences with accession number for six drug resistance genes. All these sequences were accepted in the gene bank and each sequence took the accession number (MK503659, MK559695). The current study succeeded in relying on both isolates [Pa7(MK503659) and Pa10 (MK559695)] to be standard indicators

as positive control like reference isolates to compare the results of the genetic expression of 16S rRNA *bla*$_{OXA10}$,*bla*$_{VIM}$, *aac(6')Ib, MexY, MexD and MexF* genes for other isolates based on these two isolates.

Real time PCR quantification which was applied in this study utilizes the TaqMan qPCR. Amplification of fragment of mRNA was performed with the following master amplification reaction with the program of One-Step RT-PCR: Species-specific primers and probes were designed for *bla*$_{OXA10}$, *aac(6')Ib and MexY* genes as an identification target of five (5) from (19) of *P. aeruginosa* isolates. The different sources of these isolates were distributed as follows: (1 wound, 1 burn, 1 urine, 1 sputum and 1 ear) as well as a reference gene *rps*L (201bp) for the evaluation of selected virulence gene expressions.

The expression of *bla*$_{OXA10}$ showed that the highest level of folding with an average of 4.06 was found in the case of isolates treated with Ceftazidime at the concentration of 128µg/ml. Three isolates (Pa5,Pa8 and Pa10) showed overexpression of *bla*$_{OXA10}$ with higher MIC of Ceftazidime (256-512 µg/ml).The sub-MIC of ZnO nanoparticles effectiveness on the expression of both *bla*$_{OXA10}$ and *rps*L genes in the selected target isolates showed a significant difference according to χ^2 values at $p<0.05$ among the target isolates. Treatment with ZnO NPs led to the decrease in the value of gene expression in four isolate of *bla*$_{OXA10}$ that was nearly the average (0.45) at the concentration of 325µg/ml range and the change from 0.001 to 0.13-fold in *bla*$_{OXA10}$ gene expression was due to effectiveness exposure of 325µg/ml ZnO NPs which highly affected the *bla*$_{OXA10}$ expression. On the other hand, the isolate (Pa10) was not affected due to ZnO NPs sub-MIC where the fold values were 1.04. There was also no change, no decreasing and no increasing in the expression folds which stayed in the range of one (1).

The expression of *aac(6')Ib* showed that the highest level of folding was with an average of 14.77 in the case of isolates treated with Gentamicin at the concentration of 256µg/ml. Two isolates (Pa5 and Pa10) showed overexpression of *aac(6')Ib* with the fold changes of 30.22 and 32.08 respectively. Treatment with ZnO

NPs led to the decrease in the value of gene expression in (3) isolates of *aac(6')Ib* that were 0.001,0.44 and 0.63 at the concentration of 325µg/ml. On the other hand two isolates (Pa5 and Pa7) were not affected due to ZnO NPs sub-MIC as the fold values were 2.56 and 11.36. There was also no increase in expression folds and they were not effected by ZnO NPs. Therefore, the present study could be the first study of *P. aeruginosa aac(6')Ib* gene expression.

The expression of *MexY* showed that the highest level of folding with an average of 8.16 was found in the case of isolates treated with Gentamicin at a concentration of 256µg/ml. One isolate (Pa2) showed overexpression of *MexY* with a fold change 25.52. Treatment with ZnO NPs led to a decrease in the value of gene expression in all the five isolates of *MexY* with an avarege of 0.39 at the concentration of 325µg/ml. This is due to the effective exposure of 325µg/ml ZnO NPs which highly affected the *MexY* expression.

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A list of Abbreviations

Abbreviation	Exegesis
ACC	Aminoglycoside N-acetyltransferases
AIDS	Acquired Immunodeficiency Syndrome
AMEs	Aminoglycoside modifying enzymes
ANT	Aminoglycoside nucleotidyltransferase
APH	Aminoglycoside phosphoryltransferase
cDNA	Complementary DNA
CF	Cystic Fibrosis
D.S.D.W	Deionized Sterile Distilled Water
D.W	Distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
EPS	Exopolysaccharide
ESBL	Extended-Spectrum β –Lactamase
LPS	Lipopolysaccharide
MBL	Metallo Beta-Lactamase
MDR	Multidrug-Resistant or Resistance
MIC	Minimum Inhibitor Concentration
ORFs	Open Red Frames
PBS	phosphate-buffered saline
PCR	Polymerase chain reaction
PDR	Pan-drug resistant
qRT	Quantitative reverse transcription
QS	Quarium Sensing
RNA	Ribonucleic acid
UTI	Urinary Tract Infection
WHO	World Health Organization
XDR	Extensivly-drug resistant
ZnONPs	Zinc oxide nanoparticles

Introduction

Pseudomonas aeruginosa strains, especialy multidrug-resistant, have caused serious problems in many countries, including Iraq.The increasing prevalence of nosocomial infections produced by multidrug-resistant (MDR) ,extensively drug resistant (XDR) and pandrug-resistant (PDR) *Pseudomonas aeruginosa* strains poses a grim challenge for antimicrobial therapy (El Zowalaty *et al*., 2015).

Pseudomonas aeruginosa is an opportunistic pathogen involved in many infections worldwide, such as respiratory infections, urinary tract infections, hospital-acquired pneumonia, wound and soft tissue infections and bacteremia in immunocompromised patients, including patients with thermal injuries (Morita *et al*.,2014; Weiner *et al*.,2016).

Among opportunistic pathogenic bacteria, *P. aeruginosa*, which produces distinct virulence factors, is known to be an important human pathogen, responsible for numerous infections (Livermore and Yang,1987**;** Pachori *et al*., 2019). It is Gram-negative bacilli of diverse environmental settings, and it can be isolated from various living sources.

Because of the high potency of quick adaptation, it is the most dangerous opportunistic pathogen, and it causes infections in patients suffering from cancer, Acquired Immunodeficiency Syndrome(AIDS) and cystic fibrosis (Brooks *et al*., 2016 **;** Pang *et al*., 2019). Recently, World Health Organization classified *P. aeruginosa* as one of the critical pathogens in its first published list of antibiotic-resistant priority pathogens based on the urgency of need for new antibiotics (WHO, 2017; Willyard, 2017).

P. aeruginosa infections are problematic due to its intrinsic as well as acquired resistance to many effective groups of antibiotics. Intrinsic MDR *P. aeruginosa* is attributed by limited permeability of outer membrane, production of inducible β-lactamase and Multidrug Efflux system (Mohamad *et al*.,2017). Among four MDR efflux system in *P. aeruginosa,* MexAB-OprM and MexXY-*Opr*M contribute to

intrinsic resistance whereas hyperexpression of MexCD-*Opr*J and MexEF-*Opr*N leads to acquired MDR *P. aeruginosa* (Hassuna *et al*., 2015).

In addition, *P. aeruginosa* is capable of capturing and incorporating clusters of genes, conferring antibiotic resistance and enhancing virulence. With respect to this resistance, multi-drug-resistant *P. aeruginosa* isolates have surged as a consequence of the acquisition of mobile elements such as class 1 integrons and the antibiotic resistance gene cassettes associated with them (Ebrahimpour *et al*., 2018).

β-lactamases are hydrolytic enzymes that are responsible for the resistance to β-lactam antibiotics. β-lactamases have many types including extended spectrum β-lactamases (ESBLs), AmpC β-lactamases and metallo-β-lactamases (MβLs) (Upadhyay *et al*.,2010). MBL gene is located on specific genetic elements including integrons, transposons, plasmids or on the chromosome, in which they carry genes encoding determinants of resistance to Carbapenems and other antibiotics, conferring multidrug resistance to *P. aeruginosa*. In addition, these genetic elements are transferable to other Gram-negative species, increasing the antimicrobial resistance rate and complicating the treatment of infected patients (Hong *et al*., 2015).

Genetic coded modifying enzymes like acetyltransferase (AAC), nucleotidyl transferase (ANT) and phosphotransferase (APH) are the most found methods that *Pseudomonas aeruginosa* strains are equipped with against aminoglycosides (Odumosu *et al*.,2015). One key reason for therapy failure is the increased level of antibiotic resistance among clinical *P. aeruginosa* isolates (Hanson,2013).Thus, the detection of the underlying resistance mechanisms is critical for better management of this problem.

Nanoparticles of metal oxides having a size range of 1–100 nm represent a new orientation that is increasingly being progressed for use in research and medically-care related implementation (Anbuvannan *et al*., 2015). ZnO NPs is of maximum interest because they are inexpensive to produce, safe and can be prepared easily (Jayaseelan *et al*., 2014). It has a wide range of biomedical applications like drug

delivery, anti-cancer, anti-diabetic, antibacterial, antifungal and agricultural properties(kaur *et al*. ,2015). Little is known about the antibacterial activity of ZnO as nanoparticles (Jones *et al*., 2008). In addition, ZnO is one of five zinc compounds that are presently registered as general recognized as safely by the World Health Organization (WHO) (Lee *et al*., 2017).

Current study aimed to phenotypic and molecular investigation of multidrug-resistant (MDR) *Pseudomonas aeruginosa* and measure the expression of the of beta lactams and aminoglycosides resistance genes and compare the gene expression in the present of the antibiotic, ZnONPs and in the absence of them in order to improve the role of this gene in the resistance of ***P.****aeruginosa* to antibiotics.

For this aim, the following steps were performed:

1- Isolation and identification of *Pseudomonas aeruginosa* from different clinical infections.
2- Investigations the occurrence of multi-drug resistant and antibiotic susceptibility profile in *Pseudomonas aeruginosa* isolates, as well as the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) for certain antibiotics.
3- Phenotypic detection of the of Extended-spectrum β-lactamase (ESBLs) and metallo beta-lactamase (MBLs) enzymes.
4- Molecular investigation of some genes coded for resistance to beta lactams, aminoglycosides and quinolones using PCR technique (*bla*$_{PER}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{NDM}$,*bla*$_{OXA-10,}$ *aac(6`)Ib*,*aac(3`)II* and *ant(4`)Ib*).
5- Molecular screening of *Pseudomonas aeruginosa* efflux pumps MexAB-*Opr*M, MexXY-*Opr*M, MexCD-*Opr*J and MexEF-*Opr*N.
6- Typing clinical isolates of *P. aeruginosa* by using ERIC-PCR.
7- Molecular detection of class (1) integron responsible for antibiotic resistance by using PCR technique.
8- Studying gene expression of *bla*$_{OXA-10}$, *aac(6`)Ib* and *MexY* genes using quantitative RT-PCR technique.

1. Literature Review

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a bacterium that belongs to the family «Pseudomonadaceae» (Silby *et al*., 2011). It is an aerobic Gram-negative rod that measures (0.5 to 0.8) µm by (1.5 to 3.0) µm (Lee *et al.*, 2015).*P. aeruginosa* produces pigments that inhibit the growth of other kinds of bacteria. The most important pigments are Pyocin which is blue soluble pigment in water and Pyoverdin which is green-yellowish pigment that is also known as Pseudobactin, those pigments are toxic to the host cells (Orlandi *et al.*, 2015). Preliminary *P. aeruginosa* can be identified by its specific odor *in-vitro* and by the color of the producing colonies which is mostly blue greenish. The optimum temperature for the growth of *P. aeruginosa* is 37°C, while maximum temperature of its growth is 42°C. Usually, the strains of *P. aeruginosa* are motile by means of a single polar flagellum (Brooks *et al.*, 2016). *P.aeruginosa* is widespread microorganism in natural habitats, and is possible to isolate it from the multiple environmental niches, such as: Water, soil, plants, animals and humans (Mesquita *et al.*, 2016). *P. aeruginosa* is also an important clinical agent, as this bacteria is an opportunistic pathogen that can cause wide range of acute and chronic injures and diseases in humans (Weiner *et al*.,2016).

Pseudomonas aeruginosa is one of the curses of the burn units since this bacterium is one of the most frequent source of wound and burn sepsis (Saaiq *et al.*, 2015). One of the biggest problems in treating the infections that are associated with *P. aeruginosa* is that this bacterium often prospers in clinical environments. There are many reports that refer to the detecting of multi resistant strains of *P. aeruginosa* from hospital bed rails, floors, sinks and from the hands of medical personnel. Multi drug resistant clones can remain in hospitals for many years because of the patient to patient transfer (Perez *et al.*, 2014) and for that reason *P.*

aeruginosa is one of the major nosocomial pathogens which attributes to the high percentage of patient mortality and morbidity (Pang *et al*., 2019).

Prevention or treatment of infections associated with this bacteria is a big problem mainly due to its capability of developing antibiotic resistance. Antibiotics can be expelled outside the cells by membrane transporter proteins that are called efflux pumps. Of particular interest, efflux pumps are capable of extruding out the bacterial cell a different of structurally unrelated compounds (Spengler *et al*., 2017). Efflux pumps contribute to the multidrug resistance in bacteria by expelling different types of antibiotics and chemicals such as dyes, detergents, organic solvents, biocides and metabolic products (Dreier and Ruggerone,2015).

1.2 Classification

The family Pseudomonadaceae is classified into five groups based on rRNA/DNA homology and common culture characteristics (Japoni *et al*., 2009 ; Carroll *et al*., 2016).

The scientific classification of *P. aeruginosa* is as follows:

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gamma Proteobacteria

Order: Pseudomonadales

Family: Pseudomonadaceae

Genus: *Pseudomonas*

Species: *aeruginosa*

1.3 Pathogenicity of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a successful opportunistic secondary pathogen and most predominant species in community infections ranging from superficial, mild to severe infections (Breidenstein *et al*., 2011). During hospitalization, infections are more common and varied (Kumar *et al*., 2016). Individuals at most risk include those with Immuno suppressed patients such as those with severe burns, cancer and acquired immune deficiency syndrome (AIDS) (Pang *et al*., 2019). Lung infections caused by *P. aeruginosa* can be attributed to two broad categories: (i) the high pathogenicity and adaptability of *P. aeruginosa*; and (ii) the magnitude of host defense mechanism (Williams *et al*., 2010).

Steps for an infection include some disruptions that take place in the primary physical barriers (skin or mucous membrane), or by passing them (by invasive devices) as well as an underlying dysfunction of the immune defense mechanism which is necessary as a complementary reason to accelerate the infections (Ratkai, 2011).A prior antibiotic therapy that eliminates normal flora can also provide *P. aeruginosa* to access tissue colonizing (Kumar *et al*.,2016). *P. aeruginosa* is a regular pathogen of nosocomial pneumonia, nosocomial urinary tract infections, surgical site infections, infections of severe burns and wounds, otitis externa, keratitis and folliculitis (Emami *et al*., 2015). It also causes infections of patients undergoing either chemotherapy for neoplastic diseases or some broad spectrum antibiotic therapy (Shaan and Robert,2013).

Pseudomonas aeruginosa is a notable pathogen not only because of the number and severity of *P. aeruginosa* infections, but also because of the wide range of tissues in which it causes infection (Gellatly and Hancock 2013). It is not surprising that *P. aeruginosa* infections are associated with significant morbidity and mortality due to its capacity to adapt easily to changes in the environment, to rapidly develop resistance to antibiotics, and to produce a variety of virulence factors (Ivan *et al*., 2010).

In intensive care units (ICU), *P. aeruginosa* ranks among the most dreaded of the top five microbial causing thermal burns, pulmonary, bloodstream, urinary tract, postsurgical wounds, septicemia and soft tissue infections (Veesenmeyer *et al* ., 2009). It was also the 2nd leading cause of nosocomial pneumonia (14 ~ 16%), the 3rd most common cause of urinary tract infections (7 ~ 11%), the 4th most frequently isolated pathogen in surgical site infections (8%), and the 7th leading contributor to infections of bloodstreams 2~6% (Lister *et al* ., 2009). Recently, World Health Organization classified *P. aeruginosa* as one of the critical pathogens in its first published list of antibiotic-resistant priority pathogens based on the urgency of need for new antibiotics (WHO, 2017). It's incidence found in approximately(50%) to (60%) of the persons with tenuous or immunodeficiency(Migiyama *et al* ., 2016).

The diversity of *pseudomonas* infections is due to the development of various adaptive mechanisms such as the nutritional and metabolic pathways besides the regulation of gene expression (Rayn,2011). Longitudinal studies using transcriptomic approaches have provided relevant information regarding the genetic changes undergo by *P. aeruginosa* and allowed comparing the expression of specific set of genes among patients in different periods of time (Huse *et al*., 2010; Rau *et al*., 2010).

The pathogenesis of *P. aeruginosa* opportunistic infections is diversified, as suggested by the massive number of cell-associated and extra-cellular virulence determinants; some of these factors help colonization, whereas others facilitate bacterial infestation (Holban *et al* ., 2013).

1.4 Epidemiology of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is ubiquitous in the natural environment and can adapt to habitats ranging from surface waters to disinfectants and in the humidifiers of respirators. It can multiply in distilled water, presumably by the

utilization of gaseous dissolved nutrients, but is rarely isolated from sea water (except from sewage outfalls and polluted river estuaries) (Schroth *et al*., 2018). The hospital locations, sinks, taps and drains are invariably colonized by *P.aeruginosa*. The domestic household location is infrequently contaminated, where the organism may reside in water traps (Slama *et al*., 2011).

Usual carriage by humans is rare, in healthy subjects, faecal carriage rates vary between 2% and 10%, faecal colonization appears to be passing in healthy people, and there is a quick turnover of strain types. *P. aeruginosa* dies rapidly on dry healthy skin, but in conditions of super hydration, such as in divers undergoing long term saturation dives, the frequency of skin colonization is increased and accompanied by infections, particularly otitis externa (Carroll *et al.*,2016).

In spite of the apparent ubiquity of *P.aeruginosa* in the natural environment and the vast array of potential virulence factors, the incidence of community acquired infections in healthy subjects is relatively low. The salivary carriage rate is similar in hospital patients and normal controls (approximately 5%), but skin colonization in burn patients may reach 80% by the 9^{th} day following the burn (Pirnay *et al*.,2013).

With other monitoring, *P. aeruginosa* was implicated in (16%) of nosocomial pulmonary, (12%) in urinary tract infections, (8%) in post- surgical operation contagion and (10%) in bloodstream contagion (Vitkauskien *et al* ., 2011). An estimation achieved by Centers for Disease Control and Prevention CDC in the USA showed that 51,000 healthcare-associated *P. aeruginosa* infections occur in the United States each year. More than 6,000 (13%) of these are multidrug-resistant, with roughly 400 deaths per year attributed to these infections (CDC, 2013).

Approximately, any type of hospital tools or instrument has been implicated as a reservoir for *P.aeruginosa*, including disinfectants, antiseptics, intravenous fluids

and eyewash solutions. These sources may serve as foci for the dissemination of the organism in common source outbreaks, and this usually is the result of poor sterilization (Brooks *et al*., 2016).

1.5 Genomic of *Pseudomonas aeruginosa*

The structure of the *P. aeruginosa* genome is a mosaic resulting from multiple acquisitions from different donors during its evolution, to horizontal gene transfer including the presence of genes or remnants of genes associated with mobile elements (i.e., insertion sequences, bacteriophages or plasmids) and the presence of numerous genomic islands (Bachta *et al*.,2018).

Pseudomonas aeruginosa has one of the largest genomes among bacterial human pathogens, averaging 6.6 Mbp in size (Silby *et al*., 2011). It has one chromosome, and its genetic material is rich in guanine and cytosine (G+C), in addition to the presence of extrachromosomal genetic element called plasmid (Kung *et al.*, 2010). The complete genome sequencing of a wild-type *P. aeruginosa* (PA01) strain, implement in 2000, has to outfit a great pledge of useful information, concerning not only its pathogenicity but also its probable antibacterial resistance, with 5570 open reading frames. *P.aeruginosa* genome is among the major genomes in the prokaryotic world, which encodes an unusually high attribution of proteins involved in regulation, virulence functions, and transport, which may explain the high fluctuate and adaptive capacity of this organism, and 0.3% of the total genes code for proteins complicated in antimicrobial resistance(Gimenez *et al*.,2018).

The genome is highly flexible, with 10% of genes organized in 'pathogenicity islands' (PAGI) including flexible genes coding for virulence factors. with the ability to facilely acquired large locomotive genetic elements (integrons) encoding for antimicrobial resistance genes (Stover *et al*.,2010). The genome size of *P. aeruginosa* differs from 5.2 to 7.1 Mbp**.** This degree of difference has important implications for the methods used to study the evolution and epidemiology of this

organism. It has been counseled that more than 80% of the genome are of the sequenced strain (strain PAO1) (Kung *et al*., 2010). *P. aeruginosa* decoration genome may provide to the heterogeneity of virulence. (Lee *et al*., 2016)**.**

Pseudomonas aeruginosa genomic islands have been found to possess genes encoding factors that are involved in genetic mobility and in various virulence traits such as iron uptake functions, antibiotic resistance, biofilm synthesis, type III secretion systems, toxins and adhesions that augment the ability of pathogens to survive in diverse hosts and cause of disease (Qui *et al*., 2009).

1.6 Antibiotic Resistance in *P. aeruginosa*

A general definition of antimicrobial resistance is the ability of an organism to resist the action of an antimicrobial agent to which it was previously susceptible (Pachori *et al*.,2019**)**. *Pseudomonas. aeruginosa* belongs to the family of so-called "ESKAPE" pathogens, which effectively "escape" the effects of recently available antimicrobial agents and are considered by the Infectious Diseases Society of America IDS,, as being the primacy pathogens for the urgent development of novel antibiotics and substances like-drug (Boucher *et al* ., 2009).

Pseudomonas aeruginosa has become an important and frequent opportunistic nosocomial pathogen. This organism is characterized by an innate resistance to multiple classes of antimicrobials, causing difficult-to-treat infections, which are therefore associated with significant morbidity and mortality (Mohamad *et al*.,2017).

Infection with *P. aeruginosa* is a serious clinical problem, particularly in immune compromised hosts in hospital settings (Fujitani *et al*, 2011). Moreover, the treatment of these infections is often difficult because of the limited number of effective antimicrobial agents, due to the intrinsic resistance of *P. aeruginosa* strains and their different modes of growth (Khan *et al*, 2010). This resistance reflects the synergy between the bacterium’s low outer-membrane permeability, its chromosomally encoded AmpC β-lactamase, and its broadly specific drug efflux

pump (Masuda *et al*, 2000). Furthermore, *P. aeruginosa* readily acquires resistance to most antimicrobials through mutations in its chromosomal genes and through extrachromosomal elements carrying resistance determinants (Qiu *et al*, 2009; Livermore and Yang, 1987).

The broad-spectrum resistance of *P. aeruginosa* is mainly due to a combination of different factors: (i) low outer membrane permeability (Nikaido , 2011), (ii) Presence of the inducible AmpC chromosomal ßlactamase (Lister *et al*., 2009), (iii) synergistic action of several multidrug efflux systems (Poole, 2014), and (iv) prevalence of transferable resistance determinants, in particular, carbapenem hydrolyzing enzymes (mainly metallo-ß-lactamases (MBL) (Ghasemian *et al*., 2018).

Although there are several antimicrobials (Carbapenems, Cefepime, Ceftazidime, Tobramycin and Amikacin) that continue to be effective against *P. aeruginosa,* in the last few years, the bacterium's increasing resistance to many others has been reported (Ruiz-Martinez *et al*, 2014).

Carbapenems have good antimicrobial activity against *P. aeruginosa* but the emergence and spread of acquired carbapenem resistance in this species have challenged the success of therapeutic and control efforts. Since Carbapenems, especially Imipenem, are widely used in the clinical setting (Bakhat *et al*, 2019), investigation of the molecular mechanisms leading to resistance is crucial.

From Mashhad, in Iran Imam Reza hospital Mirbagheri *et al* .(2015) isolated 131 clinical isolates of *Pseudomonas aeruginosa* to study on imipenem resistant and prevalence of bla_{VIM1} and bla_{VIM2} metallo-beta lactamases. Results of this study suggests increase in MBL-producing *P. aeruginosa* isolates as well as increase in drug impedance among these strains.

Also results of an Iraqi study by (AL-Thwani *et al* ., 2013) who isolated 28 *Pseudomonas aeruginosa* secluded from 100 burn patients in three hospitals in

Baghdad,Iraq . The antibiogram test clarified that bacterial isolates were MDR due to manifest high resistance against Ceftazidime, Ceftriaxone, Cefotaxime, Cefepime, Tobramycin and Chloramphenicol.

1.7 Multidrug Resistance *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is divided into different phenotypes based on the drug resistance patterns of the organism (Gill *et al*.,2016). Multidrug-resistant (MDR) phenotype is defined as *P. aeruginosa* which is resistant to more than one antimicrobial agent in three or more antimicrobial categories. A similar resistance to more than one antimicrobial agent in <3 antimicrobial categories is defined as drug-resistant (DR) *P. aeruginosa*. Extensively DR (XDR) phenotype is defined as *P. aeruginosa*, which is resistant to more than one antimicrobial agents in all antimicrobial categories, except in two or less. Pan-DR (PDR) phenotype is defined as a bacterium which is resistant to all antimicrobial agents in all antimicrobial categories (Magiorakos,2012).

Bacteria that are classified as XDR are epidemiologically significant due not only to their resistance to multiple antimicrobial agents, but also to their ominous likelihood of being resistant to all, or almost all, approved antimicrobial agents. In the medical literature, XDR has been used as an acronym for several different terms such as ‘extreme drug resistant’, ‘extensive drug resistant’, ‘extremely drug resistant’ and ‘extensively drug resistant (Park *et al*.,2009**)**.

MDR, XDR, and PDR phenotypes elaborate inactivating enzymes, such as extended-spectrum beta lactamases (ESBL) and metallo-β-lactamases (MBL), that make beta-lactams and Carbapenems ineffective (Ríos *et al*., 2018).

1.8 Mechanism of antibiogram in *Pseudomonas aeruginosa*

Commonly as in all other bacteria, the resistance in *P. aeruginosa* can be described in two ways either intrinsically " inherent naturalistic" whereby it's

naturally lack target sites for antibiotic and therefore the drug does not affect them due to one or a combination of factors includeing low permeability of its cell wall, genetic capacity to express a wide repertoire of resistance mechanisms and through mutation in chromosomal genes which regulate resistance genes (Al-Agamy *et al*., 2016), or it can acquire additional resistance genes from other organisms via plasmids and conjugation, transposons and transposition, bacteriophages and other diversified genetic mechanisms (Budak *et al*.,, 2012).

Pseudomonas aeruginosa resistance mechanism is classified into intrinsic or acquired resistance. The intrinsic resistance includes decreases in membrane permeability, efflux mechanism pumping the antimicrobial agents outside the cell wall and production of inactivation enzymes (Fuste *et al*.,2013).

1.8.1 Intrinsic pathway of resistance:

1.8.1.1 Membrane permeability

The outer membrane of Gram-negative bacteria including *Pseudomonas* is less permeable to hydrophobic agents due to the presence of lipopolysaccharide. The influx and uptake of antibiotics are slowed by Gram-negative outer membrane (OM) (Zgurskaya *et al*.,2015).This membrane is an asymmetric bilayer of lipopolysaccharides (LPS) and phospholipids, into which nonspecific porins and specific uptake channels are embedded.The LPS-containing bilayers are more rigid than normal bilayers, slowing passive diffusion of hydrophobic compounds, whereas narrow pores limit by size the penetration of hydrophilic drugs (Zgurskaya *et al*.,2015). Aminoglycoside and colistin interact with lipopolysaccharide leading to increases in the permeability, while beta-lactams and quinolones need to diffuse through certain porin channels. *P. aeruginosa* expresses few specific porins which allow hydrophilic molecules to pass in. Other resistance mechanism present in *P. aeruginosa* is the formation of permeability barriers (OM). Impaired penetration of different substances through the membrane (e.g. imipenem) is due to diminished expression of specific OM protein. It has been shown that OM permeabilizers such

as EDTA, increase susceptibility to antibiotics, indicating that the lack of OprD protein leads to a reduction of active antibiotic molecules capable of reaching the target penicillin-binding-proteins (Porras *et al*.,2012)**.** *Pseudomonas aeruginosa* is also set up to lack Opr D proteins. These OprD pores allow the entrance of Carbapenems. Whenever these pores are lost, Carbapenems have to face the challenges of resistance (Dantas *et al*.,2017)**.**

1.8.1.2 Efflux systems

Bacterial efflux systems capable of ejecting antimicrobials are mostly encoded by chromosomal genes and generally fall into five classes (Avrain *et al***.**, 2013) which are:

- The major facilitator superfamily (MFS).
- The ATP-binding cassette (ABC) family.
- The small multi-drug resistance (SMR) family.
- The multi-drug and toxic compound extrusion (MATE) family.
- The resistance-nodulation-division (RND) family.

P. aeruginosa expresses several efflux pumps, these pumps expel drugs together with other substances out of the bacterial cell. The efflux pump consists of three proteins:

(1) A protein transporter of the cytoplasmic membrane that uses energy in the form of proton motive force

(2) A periplasmic connective protein

(3) An outer membrane porins (Sun *et al.*, 2014).

RND family contributes significantly to intrinsic and acquired resistance to antimicrobials in a number of Gram-negative bacteria such as *P. aeruginosa* (Dreier and Ruggerone,2015).

1.8.1.2.1 RND efflux pumps

The intrinsic and acquired resistance of *P. aeruginosa* to many antibiotics is important for its ability to establish infections and cause disease in humans and animals as it will often resist treatment by antibiotics (Hoiby *et al*.,2010). The PAO1 genome encodes multiple efflux pumps of the resistance-nodulation-division (RND) type (Figure 1.1). However, *P. aeruginosa* is also able to acquire plasmids encoding genes for resistance to antibiotics that it is not intrinsically resistace, leading to clones resistant to virtually all clinically relevant antibiotics (Liu *et al*.,2016).

The pumps consist of three components: an efflux transporter in the inner membrane, an outer membrane channel and an accessory protein 14 connecting the two in the periplasm (Nikaido,2011). "RND efflux pumps often have broad substrate specificity that is not limited to antibiotics.

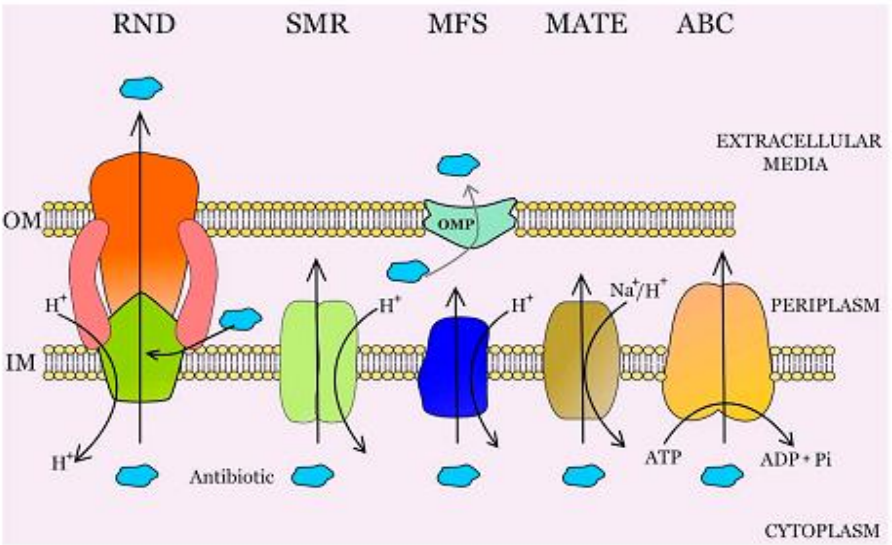


Figure (1.1) Schematic representation of the main types of bacterial efflux systems. Schematic illustration of the five major families of efflux transporters: the resistance- nodulation-division (RND) family, the small multidrug resistance (SMR) family, the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion (MATE) family and the adenosine triphosphate (ATP)-binding cassette OM: Outer membrane. OMP: Outer membrane protein(Blanco *et al*.,2016).

Four of these efflux pumps are more prevalent in *P. aeruginosa*: MexXY-*Opr*M can cause resistance to aminoglycosides, beta-lactams and fluoroquinolones; MexEF*Opr*N is related to resistance to fluoroquinolones; and MexCD-*Opr*J and MexAB-*Opr*M can cause resistance to beta- lactams and fluoroquinolones (Whalen *et al*.,2015; Shigemura *et al*.,2015).

The MexAB-*Opr*M efflux pump belongs to the superfamily of ribonucleoproteins and consists of an inner membrane (MexB), a periplasmic membrane fusion protein (MexA) and a channel-forming outer membrane protein, *Opr*M (Wu *et al*.,2008).

The multidrug efflux system MexEF-*Opr*N is produced at low levels in wild-type strains of *Pseudomonas aeruginosa*. However, in so-called *nfxC* mutants, mutational alteration of the gene *mexS* results in constitutive overexpression of the pump, along with increased resistance of the bacterium to chloramphenicol, fluoroquinolones and trimethoprim (Juarez *et al*.,2017).

The efflux pump mexXY-*Opr*M is capable of extrusion of aminoglycosides from the cytosol as well as from the periplasmic space contributing to both intrinsic and adaptive resistance (Islam, 2008).

MexXY-*Opr*M is a unique pump in *P.aeruginosa* by providing resistance to the aminoglycoside class of antimicrobials and in being inducible by many of its substrate antimicrobials (Singh *et al.*, 2017). The MexXY components are encoded by an operon under the control of an adjacent repressor gene (*mexZ*). Only the *mexXY* operon is antimicrobial-inducible, with only those agents known to target the ribosome promoting *mexXY* expression, and this is compromised by so-called ribosome protection mechanisms (Morita *et al.*,2012).

1.8.1.3 Antimicrobial inactivating enzymes:

Production of antibiotic-inactivating enzymes that break down or modify antibiotics is one of the major mechanisms of intrinsic resistance in bacteria. Many antibiotics have chemical bonds such as amides and esters that are susceptible to hydrolysis (Wright, 2005; Pang *et al*.,2019) by enzymes commonly produced by *P. aeruginosa* such as β-lactamases and aminoglycoside-modifying enzymes (Wolter and Lister,2013).

Hydrolysis of β-lactam antibiotics by β-lactamases is the most common mechanism of resistance for this class of antibacterial agents in clinically important Gram-negative bacteria figure (1-2). Because Penicillins, Cephalosporins, and Carbapenems are included in the preferred treatment regimens for many infectious diseases, the presence and characteristics of these enzymes play a critical role in the selection of appropriate therapy (Bush and Jacoby 2010). All *P. aeruginosa* strains possess the ampC gene for the inducible chromosomal β-lactamase .The β-lactams induce synthesis of a new penicillin-binding protein, PBP2a, which does not bind any β-lactam (Jawetze,2016).

Due to more sophisticated molecular approaches than were previously available, it has become increasingly easy to obtain nucleotide sequences, with their deduced amino acid sequences, for the genes encoding these enzymes in β-lactam-resistant clinical isolates. Classification of β-lactamases has traditionally been based on either the functional characteristics of the enzymes or their primary structure (Bakthavatchalam *et al*., 2016).Over-expression of the enzyme results from spontaneous mutation in the regulatory gene, ampR. over-production of the ampC β-lactamase poses a particular threat to cephalosporins. Other β-lactamases produced by *P. aeruginosa* including extended-spectrum plasmid-mediated enzymes (ESBLs) active against Penicillins and Cephalosporin (Jacoby,2009).

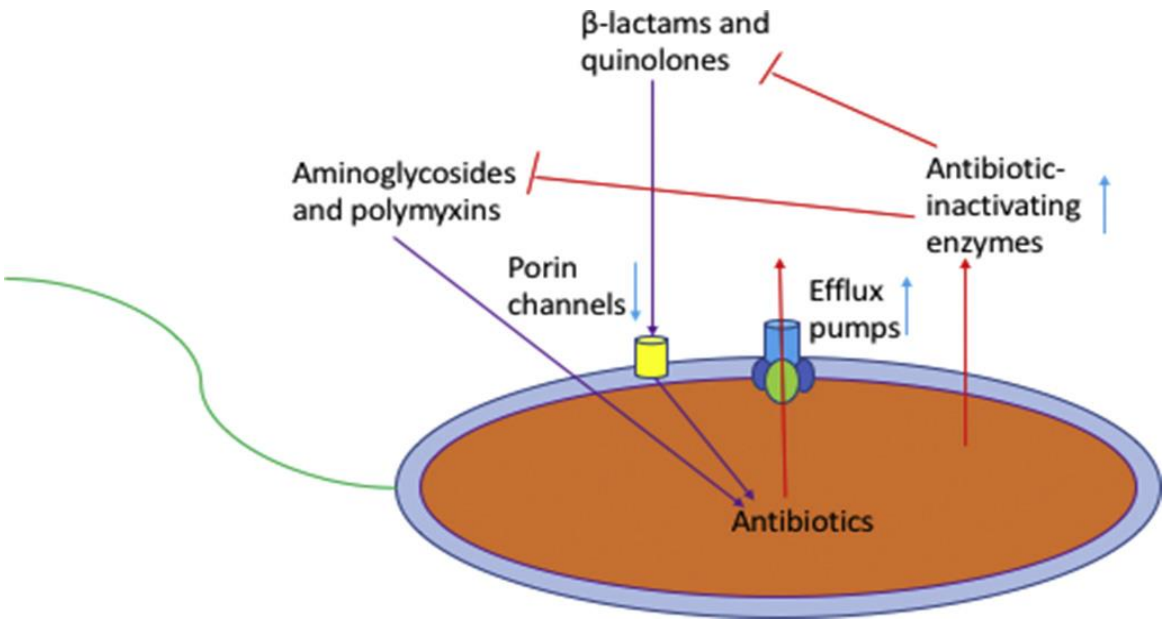


Figure (1.2) A schematic representation of the mechanisms of intrinsic antibiotic resistance in *P. aeruginosa*. The mechanisms of intrinsic antibiotic resistance possessed by *P. aeruginosa* include restricted outer-membrane permeability, efflux systems that pump antibiotics out of the cells and production of antibiotic- inactivating enzymes. Quinolones and β-lactams penetrate cell membranes through porin channels. Aminoglycosides and polymyxins promote their own uptake by interacting with *P. aeruginosa* LPS on the outer membrane.(Pang *et al*.,2019)

1.8.2 Acquired resistance:

Bacteria can gain antibiotic resistance through mutational changes or acquisition of resistance genes via horizontal gene transfer (Munita and Arias, 2016). In addition to the high level of intrinsic antibiotic resistance of *P. aeruginosa*, the acquired resistance greatly contributes to development of multidrug-resistant strains, which increases the difficulty in eradicating this microorganism and leads to more cases of persistent infections (Poole *et al*., 2011).

The main way of acquiring drug resistance in MDR *P.aeruginosa* is through acquiring plasmid. Plasmid mediated resistance has been documented by several authors (Fahimeh *et al*.,2015; Saleh and Balboula, 2017) for the genetic transfer of several drug resistance genes.

It has become increasingly clear that resistance development in *P. aeruginosa* is multifactorial, with mutations in gene encoding porins, efflux pumps, penicillin

binding proteins, chromosomal β-lactamase and aminoglycoside modifying enzymes all contributing to resistance to β-lactams, Carbapenems, Aminoglycosides and Fluoroquinolones (Porras *et al*.,2012).

Falagas and Kopterides,(2006) carried out a systemic review, and searched in pubmed for 20 years and found that the Carbapenems and Fluoroquinolones are implicated in MDR of *P.aeruginosa*. Moreover, such drug as Ampicillin makes the individuals more susceptible to *Pseudomonas* gastrointestinal infection probably resulting from suppression of normal intestinal flora, which would allow *Pseudomonas* to colonize (Campos *et al*.,2016). The increased use of carbapenems in hospitalized patients contributes to an increased selective pressure on nosocomial microorganisms, which favors the selection of bacterial subpopulations with reduced sensitivity or resistance to these antimicrobial agents (Inacio *et al*.,2014).

Extended-spectrum beta-lactamases (ESBLs) that can confer resistance to Cephalosporins are common in Enterobacteriaceae and have spread worldwide. Various class A ESBLs, such as TEM-, SHV-, VEB-, and PER-type ESBLs, and class D ESBLs such as OXA-type ESBLs have been identified in *P. aeruginosa* (Mahmoud *et al*.,2013). Antibiotic resistance is on the rise due to gene dissemination via horizontal transfer. Horizontal gene transfer via integrons is responsible for multi-drug resistance in many gram-negative bacteria particularly *P. aeruginosa* (Budak *et al*.,2012) .

1.9 Classification of β -lactamase

1.9.1. Functional Classification

The earlier method of classification of β -lactamase enzyme involves biochemical analysis of enzyme, determination of isoelectric point, determination of substrate hydrolysis, enzyme kinetics and inhibition profiles (Queenan and Bush, 2007). This functional classification of Bush and others has undergone

several revisions and currently divide β-lactamases into four functional groups (1-4). The group 2 has several subgroups that are differentiated according to group specific substrate or inhibitor profile. In this classification, Carbapenemases fall under group 2f, 2d and 3 (Queenan and Bush, 2007; Berrazeg *et al*.,2015).

1.9.2 Molecular Classification

Ambler and others have classified β-lactamases according to the amino acid sequences into four groups (A-D) (Hall and Barlow,2004). Although this classification correlates well with the functional scheme, it lacks the details concerning the enzymatic activity of the -lactamase.

Molecular classes A, C and D contain β-lactamases with serine in their active site, while group B contains metallo β-lactamases with zinc in their active sites (Kurokawa *et al*.,2003). Group B enzymes require one or two Zn2+ cations for activity and are subdivided into three groups, B1, B2 and B3 based on sequence alignments and structural analysis. Subclasses B1 and B3 have two zinc ions, whereas subclass B2 has only one zinc ion. B2 enzymes are preferentially carbapenemases whereas B1 and B3 enzymes have broad hydrolysis spectrum (Queenan and Bush, 2007; Berrazeg *et al*.,2015).

1.9.2.1 Serine β -Lactamases

1.9.2.1.1 Class A β –lactamase

Characteristic properties of these enzymes include presence of an active site serine at position 70 and presence of a disulfide bond between Cys69 and Cys238 (changes the overall shape of the active site). All the enzymes have the ability to hydrolyze Penicillins, early and extend spectrum Cephalosporins, Aztreonam as wells as Carbapenems (Bush and Bradford, 2016).

These enzymes are inhibited by clavulanic acid and tazobactam but not by EDTA. These enzymes are placed under functional 2f subgroup (Queenan and

Bush, 2007). GESβ-lactamases were originally considered as ESBL but are now classified as serine carbapenemases. Early class A carbapenemases were mostly chromosomal (SME, IMI, NMC) but recently plasmid mediated carbapenemases. (KPC and GES) are on the rise (Kurokawa *et al*.,2003; Bush and Bradford, 2016).

1.9.2.1.2 Class D β –lactamase

Class D enzymes are OXA (for Oxacillin hydrolyzing) enzymes, which are penicillinases capable of hydrolyzing Oxacillin and Cloxacillin. These serine-β-lactamases are plasmid encoded and are found commonly in *Enterobacteriaceae* and *P.aeruginosa* (Ma *et al*., 2015). These are poorly inhibited by clavulanic acid and EDTA and are known to have large amounts of variability in amino acid sequences. Currently, there are more 239 OXA enzymes, of which at least 9 are ESBLs and at least 37 are carbapenemases (Queenan and Bush, 2007). The first OXA enzyme with carbapenemase activity was observed in an *Acinetobacter baumannii* isolate from Scotland in 1985. It was originally named ARI-1 (for *Acinetobacter* resistant to imipenem) (Costa *et al.*,2009; Bush and Bradford, 2016). The enzyme was encoded on a large plasmid. Sequencing the enzyme revealed that it belonged to OXA family of β-lactamases. The enzyme was later renamed as OXA-23. Most of these enzymes are encountered in *Acinterobacter* spp. (Livermore and Woodford, 2006).

Based on amino acid homologies, OXA carbapenemases are subdivided into nine major subgroups (Bush and Bradford, 2016). OXA 23, 24 have been isolated from clinical isolates of *Acinetobacter* spp. OXA-50 has been observed as chromosomal enzymes in several *P. aeruginosa* strains, these are not expressed all the time and may not cause overt carbapenem resistance (Queenan and Bush, 2007). This plasmid borne enzyme has the highest rate of Imipenem hydrolysis rate of all the OXA carbapenemases (Walther-Rasmussen and Hiby, 2006).

OXA beta-lactamases are found mainly in *Enterobacteriaceae* and *Pseudomonas aeruginosa* and belong to the molecular D class. They are responsible for resistance to ampicillin and cefalotin and are characterized by high hydrolytic activity against oxacillin and flucloxacillin, weakly inhibited by clavulanic acid and are often resistant to ceftazidime (Farshadzadeh *et al*.,2014).

The bla_{OXA-10} belong to bla_{OXA} type extended spectrum β- lactamases (ESBLs)-genes ambler class D (Maurya *et al*.,2017). These enzyme are named OXA because they preferentially hydrolyze oxacillin and cloxacillin. These enzymes confer resistance to ceftazidime and are poorly inhibited by clavulanic acid.OXA-10 β-lactamase also possesses the ability to hydrolyze Cephalosporins, hydrolyzing Cefotaxime, Ceftriaxone, Ceftazidime and Aztreonam at low levels but sparing Cephamycins, and Carbapenems (Mirsalehian *etal*.,2010).

1.9.2.1.3 Class B Enzymes

Metallo-β -lactamases (MBLs) enzymes, characterized by their resistance to all Penicillins, Cephalosporins, β-lactamase inhibitors and Carbapenems but are susceptible to inhibition by Aztreonam and metal ion chelators (EDTA). These enzymes contain at least one Zn2+ ion in their active sites; B1 and B3 enzymes contain two Zn2+ ions, while B2 enzymes contains only one Zn2+ ion (Lee *et al*., 2005; Nakano *et al*., 2015). The first MBLs were detected in environmental and opportunistic pathogenic bacteria, *Bacillus cereus* (BCI and BCII), *Aeromonas* spp. (CphA) and *Stenotrophomonas maltophilia* (L1) as chromosomally encoded enzymes (Queenan and Bush, 2007). These bacteria also produced additional serine –β-lactamases and both the enzymes were inducible by exposure toβ-lactams (Bush and Bradford, 2016).

The most common MBL families include the VIM, IMP, GIM, SIM, SPM and NDM enzymes, which are located within a variety of integron structures, where they have been incorporated as gene cassettes. When these integrons become

associated with plasmids or transposons, transfer between bacteria is readily facilitated (Manageiro, 2011; Labarca *et al*., 2016).

Imipinemase (IMP) was first detected in a *P. aeruginosa* strain in Japan in 1988 (Gupta, 2008).The resistance gene was found on a transferable conjugative plasmid that could easily move to other *Pseudomonas* strains (Hong *et al*., 2015). This bla_{IMP} was found within integron and was present on a large plasmid (120 kb). Later, a report from Japan suggested that, *K. pneumoniae* was also found to be carrying bla_{IMP} gene (Gupta, 2008). Conjugational transfer of wide host range R-plasmids bearing the bla_{IMP} gene is the mechanism of the dissemination of gene cassette onto various Gram-negative bacterial species (Laraki *et al*., 1999; Bush and Bradford, 2016).

VIM enzymes (for Verona integron encoded metallo-β -lactamases) share the same hydrolytic spectrum the IMP-type enzymes, with less than 40% amino acid identity (Queenan and Bush, 2007; Hong *et al*.,2015).VIM-1 (Veronese imipenemase) was first to be identified in *P. aeruginosa* in Italy (Gupta, 2008). The bla_{VIM-1} gene was also integrated as a gene cassette into a class 1 integron (Lauretti *et al*., 1999). VIM-1 has also been detected in *P. putida* isolates in Italy (Queenan and Bush, 2007).

This MBL has also been reported in *E.coli* from Greece, and *K. pneumoniae* from France (Scoulica *et al*., 2014). VIM-2 was identified for the first time in *P. aeruginosa* in 1996 from southern France (Poirel *et al*.,2010). VIM-2 is closely related to VIM-1 and is encoded by a gene cassette. The gene was located on a 45 kb non conjugative plasmid.VIM- 2 producing *P. aeruginosa* has also been reported from other countries throughout the world showing its global distribution (Gupta, 2008). Some of the entero-bacterial species also show the presence of VIM type MBLs but predominantly they are seen in *P. aeruginosa* strains (Queenan and Bush, 2007).

The NDM (for New Delhi MBL) a new MBL emerged out of India and Pakistan, it was first reported in 2009 from a *K. pneumonia* isolates obtained from a Swedish patient of Indian origin, who had received medical treatment in New Delhi-India in 2007 (Yong *et al*., 2009). It has now been identified mostly in *E. coli* and *K. pneumonia* and, to a lesser extent, in other *Enterobacteriaceae* species (Yong *et al*., 2009; Nordmann *et al*., 2016). Located on a 180 kb plasmid, it expressed high-level resistance to all Penicillins, Cephalosporins, Aztreonam, Cefoxitin, Carbapenems and Ciprofloxacin. It was susceptible only to Colistin (Nordmann *et al*., 2016). Furthermore, the $bla_{\text{NDM-1}}$ gene has carried resistance to Macrolides, Aminoglycosides, Rifampicin, Sulfamethoxazole and Aztreonam, contributing to their MDR patterns. The highly resistant phenotype, coupled with the increased prevalence of these enzymes among clinical isolates makes them a major concern for public health (Hong *et al*., 2015; Nordmann *et al*., 2016).

SPM-1(Sao Paulo metallo-β-lactamase) was first isolated in Brazil in 1997 from a *P. aeruginosa* clinical isolate that was resistant to all available antibiotics except colistin .SPM is quite different from VIM and IMP, presenting the highest amino acid identity to IMP-1 (35.5%), and represents a new subfamily of MBLs(Toleman *et al*.,2002; Hong *et al*.,2015).

1.10 Other Types of β-Lactamases-Producing *P. aeruginosa*

1.10.1 Extended Spectrum β-Lactamases (ESBLs)

Extended-spectrum β-lactamases (ESBLs) are enzymes that hydrolyze penicillin, monobactam aztreonam (ATM)) and extended-spectrum cephalosporins (cefotaxime (CTX), ceftazidime (CAZ) and ceftriaxone(CRO)) (Jiang *et al.,* 2006). The clavulanic acid is β-lactamase inhibitor capable of inhibit ESBLs (Rafiee *et al.,* 2014). The extended-spectrum β-lactamases are plasmid-encoded enzymes and the major genetic groups of ESBLs are TEM, SHV, PER and CTX-M that mediate resistance to wide range of antibiotics' generation (Peymani *et al.,* 2017).

Many *P.aeruginosa* strains produce different classes of ESBLs that enable bacterium to stand against extended spectrum Cephalosporins. Plasmids carrying CTX-Mβ-lactamases are widely distributed in clinical isolates belonging to the *Enterobacteriaceae* (Livermore, 2006). The CTX-M types are widely spread in the world (Shacheraghi *et al*., 2010). ESBLs members of the OXA family (OXA-18 and derivatives of OXA-2 and OXA10) are comparatively rare, and they found mostly in *P. aeruginosa* (Bokaeian *et al*.,2015). The VEB-1enzyme is typically plasmid born in *Enterobacteriaceae* but chromosomal in *P. aeruginosa* and *A. baumannii* (Poirel *et al*., 2010).The plasmids mediated PER -βlactamase have been described but are uncommon and have been first identified in *P.aeruginosa* (Kiratisin *et al*., 2018).

Unfortunately, the emergence of extended-spectrum β-lactamases (ESBLs) *P. aeruginosa,* among hospitalized patients in burn units is life-threating because the burn patient's infection is one of the most complicated issue since these strains are multidrug-resistant and challenging to treat (Radan *et al.*, 2016). So it is obvious that using PCR in the detection of TEM, SHV, CTX and AmpC genes in ESBL-producing bacteria and their pattern of antimicrobial resistance is quite important; For the establishment of the appropriate antimicrobial therapy and for assessment and control of the spread of drug resistant *P. aeruginosa* (Bokaeian *et al.,* 2015).

1.11 *Pseudomonas aeruginosa* Rrsistance to Aminoglycosides

Aminoglycosides are a group of clinically important, broad-spectrum antibiotics that inhibit protein biosynthesis in bacteria by selectively binding to the A-site decoding region of the bacterial 16S rRNA within the 30S ribosomal subunit causing mistranslation of mRNA or premature termination of protein synthesis (Teixeira *et al*., 2016). Resistance to aminoglycoside may be linked both to chromosomal mutations and acquisition of resistance genes located on mobile genetic elements, such as plasmids, integrons and transposons (Lister *et al*.,2009). From variety of plasmid-mediated aminoglycoside resistance mechanisms, the

most commonly encountered is the production of aminoglycoside-modifying enzymes (Tada *et al*.,2013). These modifying enzymes include aminoglycoside phosphoryl transferase (APH), aminoglycoside acetyl transferase (AAC), and aminoglycoside nucleotidyl transferase (ANT) (Woegerbauer *et al*.,2014).

Genetic coded modifying enzymes like acetyltransferase (AAC), nucleotidyltransferase (ANT) and phosphotransferase (APH) are the most found methods that *Pseudomonas aeruginosa* strains are equipped against aminoglycosides (Odumosu *et al*.,2015).

1.11.1 Aminoglycoside modifying enzymes (AME)

The major mechanism of aminoglycoside resistance in clinical isolates of both Gram negative and positive bacteria is the enzymatic modification of amino- or hydroxyl-groups of the aminoglycosides (Vakulenko & Mobashery, 2003; Chen and Munchie,2014).Modifying enzymes of aminoglycosides reduced or abolished binding of the aminoglycoside molecule to the ribosome caused failure in trigging energy-dependent phase II (Odumosu *et al*.,2015). AMEs are often plasmids encoded but are also associated with transposable elements (Tada *et al*.,2013). As part of mobilizable or conjugative plasmids, natural transformation or transduction results in the ability of this resistance mechanism to reach virtually all bacterial types (Ramirez and Tolmasky, 2010). There are three classes of AMEs: ATP-dependent AG nucleotidyltransferases (ANTs) which transfer a nucleotide triphosphate moiety to a hydroxyl group, leading to the transfer of the adenyl moiety to the aminoglycoside, ATP (and/or GTP)-dependent AG phosphotransferases (APHs) which transfer phosphoryl group from ATP to a hydroxyl group in the antibiotic and acetyle CoA-dependent AG acetyltransferases (AACs) which transfer acetyl group from acetyl-CoA to an amino group (Zaunbrecher *et al.,* 2009). All classes of aminoglycoside-inactivating enzymes have been reported in *P. aeruginosa* (Odumosu *et al*.,2015).Three families of enzymes are identified including:

1.11.1.1Aminoglycoside N-acetyltransferases (AACs)

Aminoglycoside acetyltransferases are the largest group of aminoglycoside-modifying enzymes, which selectively transfer an acetyl group from acetyl-CoA to one of the several amine functions present in aminoglycosides (Magalhaes & Blanchard, 2009). There are four major classes of AACs: AAC (1), AAC (3), AAC (2′), and AAC (6′), designated according to the site of acetylation (Haldorsen, 2011).

AAC (6') enzymes are the most common enzymes among the AACs, and are present in both Gram-negative and Gram-positive bacteria. There are two main subclasses; AAC (6')-I and AAC (6')-II. The first subclass, AAC (6′)-Ib shows activity against gentamicin and amikacin, and is probably the most clinically relevant acetyltransferase.The second one AAC (6')-II shows high activity against all three forms of gentamicin, but not amikacin (Ramirez and Tolmasky, 2010; Tada *et al*.,2013).

AAC (6')-Ib is the predominant type in bacteria. Shaw *et al*. (1993) found that among those Gram-negative isolates producing the AAC(6') resistance profile, 70.6% possess the AAC(6')-Ib gene, which can be found in gram-negative belonging to the genus *Acinetobacter, Enterobacteriaceae, Pseudomonadaceae* and to the *Vibrionaceae* (Tolmasky, 2007).The reason why the AAC(6)-Ib is so widely distributed among bacteria is that the gene for the enzyme is commonly found on mobile genetic elements, which facilitates its rapid transfer (Vakulenko and Mobashery, 2003). The AAC (3) enzymes are the second largest group of aminoglycoside acetyl transferases. There are at least nine recognized subclasses of AAC (3) enzymes described, all of them in gram-negatives (Haldorsen, 2011).

AAC(3)-I includes five enzymes (AAC(3)-Ia, AAC(3)-Ib, AAC(3)-Ic, AAC(3)-Id and AAC(3)-Ie) and confers resistance to gentamicin and other aminoglycosides . These enzymes are present in a large number of *Enterobacteriaceae* and other

Gram-negative clinical isolates and they are all found encoded as part of gene cassettes in integrons (Haldorsen, 2011; Teixeira *et al*.,2016).

1.11.1.2 Aminoglycoside O-phosphotransferases (APHs):

The APH class of enzymes is the second-largest group of aminoglycoside-modifying enzymes. These enzymes catalyze the transfer of the γ-phosphoryl group from ATP to hydroxyl groups on aminoglycoside molecules (Magalhaes and Blanchard, 2009). The genes encoding these enzymes are often found on multidrug-resistance R plasmids, transposons, and integrons (Houghton *et al.*, 2010).

Almost all phosphoryl transferases of *P.aeruginosa* act in the 3 position APH (3) of the aminoglycoside molecule (Pool, 2011), and it is the largest group of APH enzymes which can be divided to seven sub-classes I-VII (Haldorsen, 2011). However, they have less clinical importance because of the fact that they inactivate aminoglycosides and not routinely used for the treatment of *P. aeruginosa* infections such as kanamycin and neomycin (Poole, 2005). The enzymes of this family that inactivate anti -pseudomonal aminoglycosides are APH (3)-VI, APH (2) and APH (3)-IIb. Despite being reported in some cases, these enzymes remain rare for clinical *P. aeruginosa* isolates (Pool, 2011).

1.11.1.3 Aminoglycoside O-nucleotidyltransferases (ANTs):

They represent the smallest family of aminoglycoside-modifying enzymes, with only ten enzymes identified (Houghton *et al.,* 2010). ANT are mediate inactivation of aminoglycosides by catalyzing the transfer of an adenosine monophospate (AMP) group from the donor substrate adenosine triphosphate (ATP) to hydroxyl group in the aminoglycoside molecule (Rumerz and Tulmasky, 2010). These enzymes are able to catalyze an O-adenylation reaction between ATP and aminoglycoside in the presence of Mg2+ ions (Houghton *et al.,* 2010). There

are five classes of ANTs that catalyze adenlylation at the 6 ANT(6), 9ANT(9), 4 ANT(4), 2 ANT(2), and 3 ANT(3) positions (Haldorsen, 2011).

The ANT (4) kanamycin nucleotidyltransferase was originally isolated from clinical isolates of *Staphylococcus aureus* in 1976, adenylylating the 4″-hydroxyl group of kanamycin (Goffic *et al.*, 1976). The enzyme can utilize ATP, GTP, or UTP as the nucleotide substrate and can inactivate a wide range of aminoglycosides including kanamycins A, B, and C, gentamicin A, amikacin, tobramycin, and neomycins B and C (Chen and Munchie,2014).

ANT (4) includes two subclasses, I and II. The ANT (4)-I gene has been found in plasmids in Gram-positive bacteria, while ANT (4)-II enzymes have been identified in Gram negative bacteria. Two ANT (4)-II enzymes have been described; ANT (4)-IIa was identified encoded on plasmids from *Pseudomonas* and Enterobacteriaceae and ANT (4)-IIb was encoded in *P.aeruginosa* transposon (Rumerz and Tulmasky2010).

1.12 Integrons

Integrons are a segment of dsDNA that play a major role in bacterial adaptation and evolution. These genetic determinants are known by the presence of three necessary apparatuses: an integrase (*intI* gene), *Pc* (a promoter) and *attI* (a recombination site) Figure (1.3). These elements are able to acquire gene cassettes, which can carry antibiotic resistance factors, via site-specific recombination mechanism (Akrami *et al*.,2019).

Integrons, as mobile genetic elements, are primary sources of gene cassettes and disseminate antibiotic resistance genes within the microbial populations. Mobile integrons capture gene cassettes from the environment and incorporate them in the variable regions of integron by a site-specific integrase. More than 130 different cassettes have been characterized within integrons. There are more than nine classes of integrons, in which class I integrons are mostly and commonly found in

nosocomial and community environments, followed by class II and III (Khosravi *et al*., 2011; Zeighami *et al*., 2015).

They are unusual gene acquisition elements that were first identified and characterized by Stokes and Hall in 1987. The importance of integrons in the acquisition of resistance genes was recognized in the late 1980 (Stokes and Hall, 1989). A study demonstrated that most of the integrons that have been sequenced and characterized contain at least one acquired resistance gene (Fluit and Schmitz, 2004).

Two main groups of integrons are identified including: mobile integrons (MIs) and chromosomal integrons (CIs). CIs are found in marine bacterial chromosome, such as Vibrio species. The other name for this class is super-integrons (SIs), because they can carry more than 200 cassettes and usually encode proteins with unknown function (Hocquet *et al*.,2012). On the other hand, CIs can carry a numerous number of gene cassettes, which are commonly not involved in drug resistance. Mobile integrons(MIs) are located on the mobile genetic elements (MGEs) such as plasmids and transposons, and carry only a few cassettes. MIs can carry antibiotic resistance cassettes, because of this function; they called resistance integrons (RIs) (Escudero *et al*.,2018).

The number of gene cassettes can vary in an integron but the highest number of gene cassettes observed so far is eight (Escudero *et al*.,2018). Genes carried by integrons usually encode multiple resistance mechanisms such as resistance to beta-lactams, aminoglycosides and other antimicrobial agents (Elbourne and Hall, 2006; Xu *et al*., 2018). On the basis of integrase sequences, at least five classes of integrons have been described. In *P. aeruginosa*, the majority of integrons belong to class 1, in which antibiotic resistance gene cassettes are particularly common (Akrami *et al*.,2019). Integrons by themselves are not mobile. Rather, they achieve mobility only when linked to an existing mobile genetic element. Common mobile genetic element carriers on which integrons can be found are conjugative plasmids and transposons (Stokes *et al*., 1997).Owing to their ability to capture and ensure

the expression of multiple different resistance gene cassettes, integrons are particularly dominant contributors to the development of multidrug-resistant *P. aeruginosa* strains (Lister *et al*., 2009; Escudero *et al*.,2018). In addition to MBLs and aminoglycoside resistant genes, other integron-associated β-lactamases have also been implicated in *P. aeruginosa* outbreaks in hospital settings worldwide (Labbate *et al*., 2009; Zeighami *et al*., 2015).

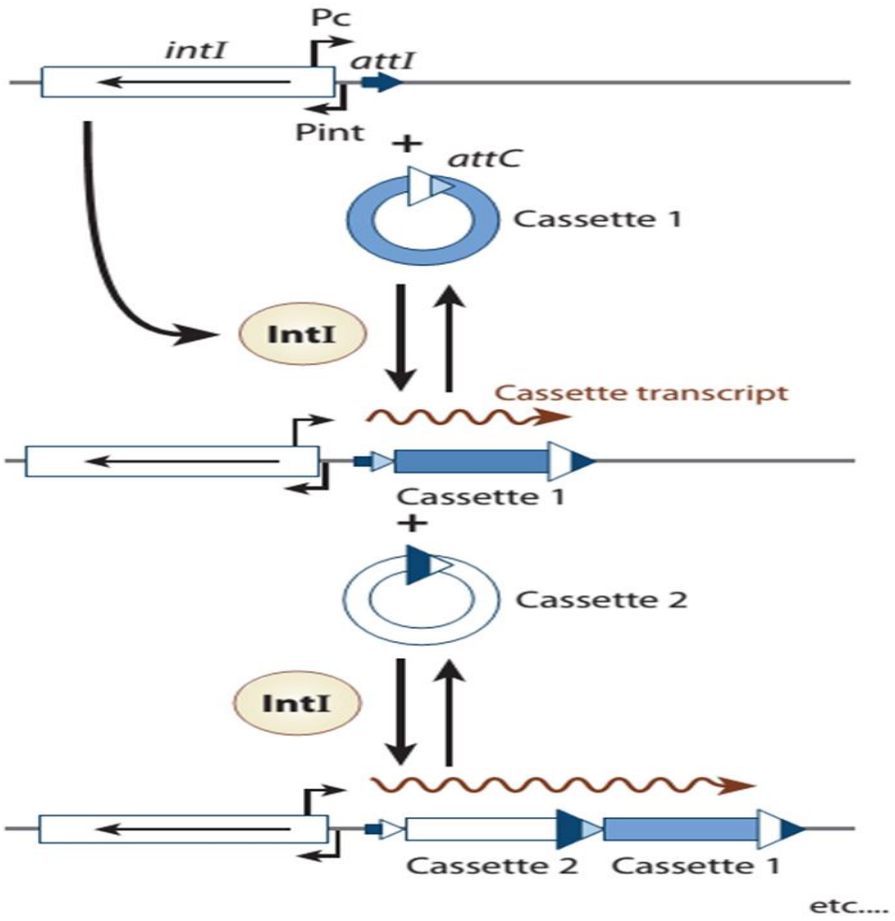


Figure (1.3): Integron-mediated gene capture and model for cassette exchange. Outline of the process by which circular gene cassettes are repeatedly inserted at the specific *attI* site in an integron downstream of the strong promoter Pc. *intI*, integrase encoding gene; IntI, integrase IntI. (Cambray *et al*.,2010).

1.12.1 The Cassette Array

Successive integration at the *attI* site results in the streamlined assembly of different gene cassette arrays (Lin *et al*.,2015). This cassette array constitutes the variable parts of the integron. Gene cassettes are minimal functional elements intended to be mobilized by the integrase of integrons. They are generally

constituted by a single open reading frame (ORF) immediately followed by a recombination site termed *attC* (formerly called 59-base element), which is specifically recognized by IntI. As mentioned above, cassette borne genes are most generally promoterless, and their expression is hence conditioned by the proximity of an external promoter, essentially Pc. Accordingly, the ORFs in cassettes are usually oriented toward the *attC* site. The excision of cassettes by the IntI integrase leads to non replicative, covalently closed circular intermediates (Cambray *et al*.,2010; Escudero *et al*.,2018).

1.13 Nano-Particles (NPs)

The word "Nano" is derived from Latin, meaning dwarf. Nano size refers to one thousand millionth of a particular unit, therefore, nanometer means one thousand millionth of a meter (i.e. 1nm = 10^{-9} m) (Gleiter,2000). Nanotechnology is defined by Professor Norio Taniguchi in 1974, as a multidisciplinary science that covers a diverse area of research and technology in chemistry, physics and biology (ENPs)(Uskokovic,2008).

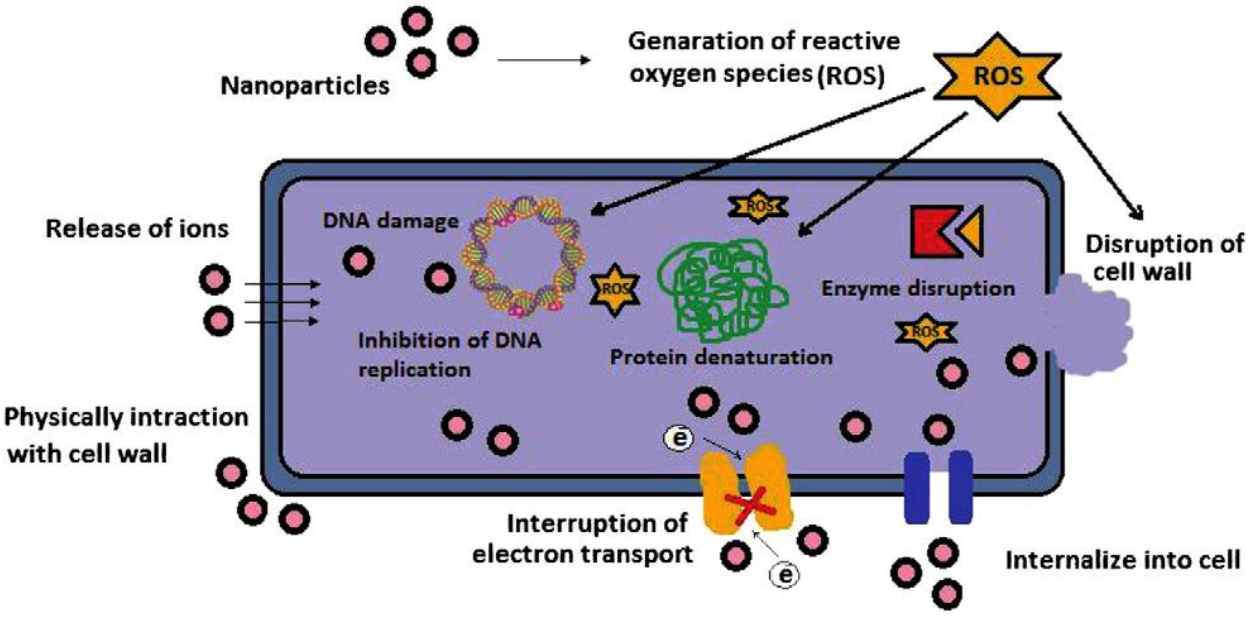
Nanotechnology is an important field of modern research dealing with synthesis, strategy and manipulation of particle structure ranging from approximately 1 to 100 nm in size. Within this size range all the properties (chemical, physical and biological) changes in both individual atoms/molecules and their corresponding bulk are investigated (Ghanbarzadeh and Almasi, 2012).

Nanoparticles are usually defined as those materials which have one or more external dimensions in the nanoscale (1 to 100 nm), that exhibit new or enhanced size-dependent properties compared to the larger particles of the same substance (Shah and Shah, 2013).

Novel applications of nanoparticles are growing rapidly on various fronts due to their enhanced properties based on size, distribution and morphology. It is swiftly gaining renovation in a large number of fields such as health care, cosmetics, biomedical, food and feed, drug-gene delivery, environment, optics, chemical

industries, electronics, energy science, catalysis, light emitters, single electron transistors and photo-electrochemical applications (Ghanbarzadeh and Almasi, 2012; Madhumitha *et al*., 2016).

The exact mechanisms of antibacterial activity of NPs are not fully understood. However, many studies suggested the following mechanisms of antibacterial effects of NPs. First, NPs can attach to the bacterial cell membrane by electrostatic interaction and can break the integrity of bacterial membrane (Thill *et al*, 2006). Bacterial cell wall is designed to provide rigidity, strength, and shape, and protect the cell from burst and mechanical damage (Azeredo,2013).The structure and composition of bacterial cell wall can be grouped into two types: Gram positive(+ve) and Gram negative (ve-).The structure of the cell wall plays a crucial role in the effect of NPs on bacteria figure(1.4) . properties of bacterial cell wall plays a key role in diffusion of NPs inside the biofilm matrixes (Baek and An, 2011).



Figure(1.4)Various mechanisms of antimicrobial activity of the metal nanoparticles(Dizaj *et al*., 2014).

1.13.1 Zinc Nano particles (ZnO NPs)

ZnO is an n-type semiconducting metal oxide. It is a wide range of applicability in the field of electronics, optics, and biomedical systems (Anbuvannan *et al*., 2015). Several types of inorganic metal oxides have been synthesized and remained in recent studies like TiO2, CuO, and ZnO. Of all these metal oxides, ZnO NPs is of maximum interest because they are inexpensive to produce, safe and can be prepared easily (Jayaseelan *et al*., 2012). US-Food and Drug Administration (FDA) has enlisted ZnO as GRAS (generally recognized as safe) metal oxide and also used as food additive as in the fortification of cereal-based foods (Xie *et al*., 2011; Sabir *et al*., 2014). ZnO NPs exhibit UV filtering properties, anti-inflammatory and wound healing (Mirzaei and Darroudi, 2017). It has a wide range of biomedical applications like drug delivery, anti-cancer, anti-diabetic, antibacterial, antifungal and agricultural properties (Jain *et al*., 2014).

Although ZnO is used for targeted drug delivery, it still has the limitation of cytotoxicity which is yet to be resolved (Ma *et al*., 2013). Biosynthesis ZnO NPs have a very strong antibacterial effect at a very low concentration of gram negative and gram positive bacteria as confirmed by the studies. They have shown strong antibacterial effect than the ZnO NPs synthesized chemically (Vimala *et al*., 2014; Madhumitha *et al*., 2016). ZnO NPs have been reported in different morphological structure such as nanoflake, nanoflower, nanobelt, nanorod and nanowire, to be utilized as antimicrobial agents to reduce multidrug resistant bacteria (Elkady *et al*., 2015; Agarwal *et al*., 2017).

The toxicity behavior of ZnO NPs can be assessed by a number of *in vitro* and *in vivo* methods. The *in vitro* research is conducted on cell cultures. Cell culture assays are used as a prescreening tool to understand the biological effects of NPs activity, their toxicity and mechanism of action. A few inorganic and many synthesized polymeric NM have been shown to have different levels of biocompatibility (Beyth *et al*., 2015).The World Health Organization(WHO) has shown that ZnO nanoparticles have no effect on human cells at different

concentrations even the high, which was detected by numerous tests and assays (Lee *et al*., 2017).

1.13.2 Role of metal NPs on changing microbial gene expression

The effect of metal nanoparticles on microorganisms DNA replication and gene expression takes a diversified potentiality ranging from highest pivotal changing up to stopping genetic expression for targeted genes, to marginal or functionally impalpable as shown by Sharma *et al*., (2009). Several methods have been developed over the years to present the relative gene expression. The efficiency correction method calculates the relative expression ratio from the real-time PCR efficiencies and the C_T according to Schmittgen and Livak (2008). The study of Shakerimoghaddam *et al*. (2017) aimed to investigate the effect of ZnONPs10-30nm in size on biofilm formation and expression of the *flu* gene encoded to Antigen 43(Ag43) surface protein has a role in processes of biofilm formation in uro pathogenic *E. coli* strains. The conclusion evidenced that the sub-MIC of ZnO NPs reduces biofilm formation and *flu* gene expression in UPEC isolates.

1.14 Enterobacterial Repetitive Intergenic Consensus (ERIC) Typing of *P. aeruginosa*

Bacterial typing has a main role of clonal relatedness determination between a microbial strains, thus it is essential to determine the routes and source of infections; as well as the relationships between microbial isolates from different source; confirm the outbreaks; trace crosstransmission of healthcare associated pathogens; assess the effectiveness of control measures; recognize particularly virulent strains; additionally bacterial typing has greatly contributed to increase the effectiveness of surveillance systems, also has provided important clues to public health control strategies (Perez-Losada *et al*., 2013).

Due to the plasticity of the phenotypic characteristics, molecular techniques (which are inherently more stable than phenotypic characteristics) have gained

popularity for strain differentiation and epidemiological studies of many organisms (Deschaght *et al*., 2011).

Molecular typing of microbial pathogens is of pivotal importance in the elucidation of transmission routes. The detailed genetic analysis at the species level gives insights into the variability within a bacterial population and generates evidence on genome plasticity and evolution, which in turn leads to bacterial adaptation to various environmental conditions (Al-Zahrani *et al*., 2012). For the last two decades, PCR-based genotyping methods have played an important role in bacterial typing schemes. Molecular researches have been performed to study diversity among *P. aeruginosa* strains, polymorphism of some of its genes and also genetic comparison of *P. aeruginosa* isolates from different hosts and environments (Onasanya *et al*., 2010 ; Al-Zahrani *et al*., 2012).

Genetic typing methods have been found to be more discriminatory than phenotypic methods for typing *P. aeruginosa* isolates (Al-Shammary *et al*.,2013). ERIC is used widely in Gram-negative enteric bacteria. Imperfect palindromes with 127 bp that occur in multiple copies in the genomes of enteric bacteria and vibrios are ERIC-PCR sequences (Wilson & Sharp, 2016).

Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) may be a useful tool for the typing of *P. aeruginosa* isolates. One of the widely used repetitive DNA elements in PCR based genotyping methods, is the ERIC sequences common to Gram-negative enteric bacteria (Khosravi *et al*.,2016). ERIC sequences may offer greater potential as an example for the study of the evolution of bacterial interspersed repetitive sequences because they are longer and thus more informative in comparative analyses and are found in a wider range of species.

1.15 Multiplex PCR

Whereas standard PCR usually uses one pair of primers to amplify a specific sequence, multiplex PCR allows the simultaneous amplification of more than one target sequence in a single reaction by using a set of primers (Rodriguez-Lazaro and Hernandez, 2013). This saves considerable time and effort, and decreases the number of reactions that need to be performed to detect the desired targets in the sample. The presence of many PCR primers in a single tube can cause some problems, such as increased formation of mis primed PCR products,primer dimers and the amplification discrimination of longer DNA fragments (Atlas and Bej, 1994; Ellington *et al*.,2007). For multiplex PCR, primers should be chosen with similar annealing temperatures. The lengths of amplified products should be similar; as large differences in the lengths of the target DNAs may favour the amplification of shorter targets over the longer ones, which results in differential yields of the amplified products (Rodriguez-Lazaro and Hernandez, 2013).

1.16 Real-time PCR

Real-time PCR follows the same principle as conventional PCR but the product is detected in real time using fluorophores (Mackay *et al*.,2002). There are different kinds of techniques that can be used. One is the SYBR® Green technique which uses a DNA binding dye that binds to double stranded DNA (dsDNA) and emits green light after the complex formation figure (1.4). After each cycle, there will be more products in the form of dsDNA which will increase the fluorescence (Sigma-Aldrich,2015). One problem with this technique is that the dye will bind to all dsDNA in the solution, including undesired primer-dimers and other by-products.

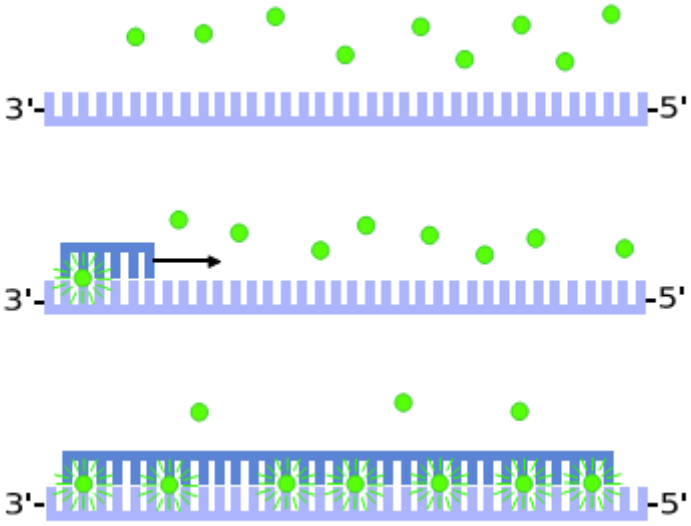


Figure 1.5. The basic theory of real-time PCR using the SYBR® Green technique. The DNA binding dye is binding to the newly formed double stranded DNA and starts emitting green light. After every cycle there will be more double stranded DNA which will increase the fluorescence.

Another technique is the fluorescent reporter probe method where a probe with a fluorophore reporter at the 5’ end and a quencher at the 3’ end are added to the PCR mix (Sigma-Aldrich,2015). The quencher absorbs the excitation energy from the reporter if they are in a close proximity. When the probe binds to the DNA fragment, the polymerase can degrade the probe base by base thanks to its 5’-to-3’ exonuclease activity. This breaks the proximity between the reporter and the quencher resulting in detectable fluorescence figure (1.5). When the target molecule is amplified, so is the fluorescence. One of the advantages with this technique is that it only detects the DNA containing the probe sequence and thus circumvents the problem with binding to all dsDNA. Another advantage is that the method can be changed into a multiplex assay using unique probes with different fluorophores. To make the detection easy and visible the selected fluorescent reporters should have no spectral overlap (David and Marta 2013).

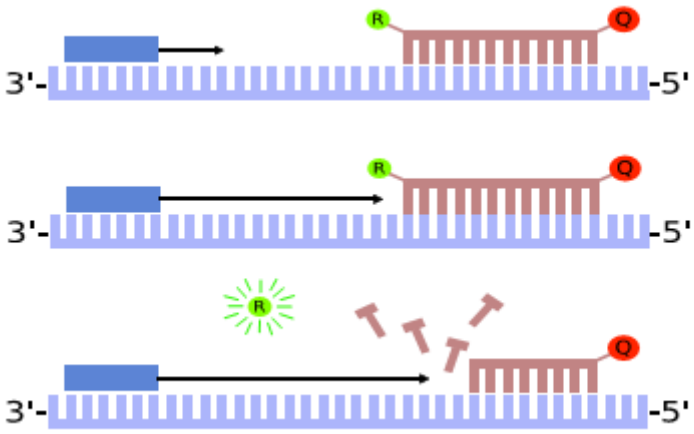


Figure 1.6 The basic theory of real-time PCR using the fluorescent reporter probe method. The primers and probe, with a fluorescent reporter (R) and a quencher (Q), bind to the DNA template. As long as the quencher is in close proximity to the reporter it absorbs the emitted fluorescence. When the polymerase elongates along the template and reaches the probe it starts degrading it and the proximity between reporter and quencher is lost, which results in detectable fluorescence.

Quantitative real-time polymerase chain reaction (qRT-PCR) is a reliable technique for quantifying mRNA levels when normalized by a stable reference gene/s (Alqarni *et al*.,2016). qRT-PCR has become the method of choice to quantify small amounts of RNA molecules owing to its high sensitivity, specificity, reproducibility and accuracy. Reliability of qRT-PCR analysis is dependent on the choice of appropriate reference genes (Costaglioli *et al*.,2014). Design of primers and probes is one of the most crucial factors affecting the success and quality of quantitative real-time PCR (qPCR) analyses, since an accurate and reliable quantification depends on using efficient primers and probes(Rodriguez et al.,2015).

Design of primers and probes should meet several criteria to find potential primers and probes for specific qPCR assays. The formation of primer-dimers and other non-specific products should be avoided or reduced. This factor is especially important when designing primers for SYBR® Green protocols but also in designing probes to ensure specificity of the developed qPCR protocol (Rodriguez *et al*.,2015).

2. Materials and Methods

2.1 Materials

2.1.1 Devices and instruments

All the devices and instruments being employed are listed in the Table (2-1).

Table (2-1). Devices and Instruments.

Devices and instruments	company	origin
Autoclave	GEMMY	Taiwan
Centrifuge	Fisher Scientific	USA
Cooling Shaker incubator	Sartorius	Germany
Distillatar	Gallenkamp	England
Eppendorf tubes	Sterellin Ltd	UK
Gel Imaging System	Major Science	Taiwan
Hot plate magnetic stirrer	TJ lassco	India
Incubator	Memmert	Germany
Inoculating loop	John Bolton	UK
Laminner Flow Hood	Lab Tech	Korea
Light Microscope	Olympus	Japan
Mic qPCR Cycler	Bio Molecular System	Australia
Micropipettes different sizes	Brand	USA
Micro spin Centrifuge	My Fugene	China
Microcentrifuge	Eppndrof	Germany
Microwave Oven	GOSONIC	China
Millipore Filters (0.22µ)	Millipore Corp.	Germany
Electrophoresis System	Thermo	USA
pH meter	Hana	Italy
Power supply	Bio-Fisher $_{GmbH}$	Germany
Quantus Florometer	Promega	USA
Refrigerator	Ariston	Italy
Sensitive balance	OHAUS-PioNEER	USA
Sterilized cotton swabs	Sterellin Ltd.	UK
Swab with media	Sterellin Ltd.	UK
Tips	Sterellin Ltd.	UK
Thermo Cycler	BioRad	USA
Ultrasonic homogenizer	Hielscher	Germany
UV- transilluminator Desktop Gel	OPTIMA	Japan

Imager(Scope21)		
VITEK-2 system	BioMerieux	France
Vortex Mixer	Labcoo	Germany
Water bath	Memmert	Germany

2.1.2 Chemicals, Reagents and Biosubstances

The important materials and substances in current study were sorted in Table (2-2) .

Table (2-2) Materials , Substances and Kits .

Materials	**Company and origin**
Absolute Ethanol and Isopropanol	ROMIL pure chemistry, UK
Agarose, Ethidium Bromide Solution(10mg/ml), Nuclease Free Water, TBE 10X, Quantiflor dsDNA System.	Promega, USA
Catalase Reagent	Analar – England
CetriNix Supplement (FD029)	Himedia –India
Chloroform	LiChrosolv, Germany
Ethylene diamine tetra-acetic acid (EDTA)	Fluka – Switzerland
Glacial acetic acid	BDH – England
Glucose and Sucrose	Oxoid - England
Glycerol	Fluka - Switzerland
Gram,s Stain Kit	Syrbio – Syria
Human blood	Diyala blood bank
Oxidase Reagent: Oxidase indicator [N,N,N,N-tetramethyl-ρ-phenylenediamine dihydr chloride %1]	Fluka – Switzerland
Sodium chloride NaCl, Sodium hydroxide NaOH, Hydrochloric acid HCl	BDH - U.K
Standard MacFarland's solution(0.5)	Bio Mérieux-France
ZnO NPs powder	Nano shell – USA

2.1.3 Kits and Materials were used in PCR and electrophoresis

The following table illustrates kits and materials were used for PCR and electrophoresis.

Table (2-3): Different kits and materials used in PCR and electrophoresis

Kits and Materials	Components of the kit	Company/origin
PCR kit (Master Mix)	Go Taq ® Green Master Mix, 2xpH equal to 8.5	Promega /USA
Blue\orange 6X loading dye	supplied with these markers have a composition of 15% Ficol, 0.03% xylene cyanole, 0.4% orange G,10mM Tris-HCl (pH=7.5) and 50mM EDTA.	Promega /USA
Wizard Genomic DNA Purification Kit	Following items are available separately: Cell Lysis Solution, Nuclei Lysis Solution, Protein Precipitation Solution, DNA Rehydration Solution and RNase A(4mg/ml).	Promega /USA
General RNA Extraction Kit	-	Promega /USA
Real Time PCR kit	GoTag qPCR Master Mix, GoTaq® 1-Step RT-qPCR System , $MgCL_2$, Nuclease Free Water, Quantiflor RNA System.	Promega /USA
TRIzol Reagent	-	Thermo Scientific, USA
Ladder	1500bp DNA Marker	Promega /USA

2.1.4 Bacterial culture media

Bacteriological media were used in recent study are schedule in table (2-4).

Table(2-4): Ready-made bacterial media .

Media	Company and origin
Agar-Agar	Himedia - India
Blood agar base	Oxoid - England
Brain heart infusion agar(BHIA)	Oxoid - England
Brain heart infusion broth(BHIB)	Oxoid - England
MacConkey agar	Himedia –India
Muller Hinton agar(MHA)	Oxoid - England
Nutrient agar(N.A)	Himedia - India
Nutrient broth(N.B)	Himedia –India
Pseudomonas Agar Base	Himedia –India
Triple sugar iron agar(TSI)	Himedia –India

2.1.5 Antibiotics

Antibiotic powders that were used in current study are listed in table (2-5) while antibiotic discs are listed in table (2-6).

Table (2-5): Antibiotic powders

Ceftazidime	AL-Razi center for production of diagnostic kits (Iraq).
Gentamicin	Siga-Aldrich / USA
Imipenem 25mg	Siga-Aldrich / USA

Table (2-6): Antibiotics items were used in the current study

Antibiotic			Con. per disk in µg	Symbol code	Manufacture company and Origin
Class		Type			
1	Penicillins	Pipracilin	100	PRL	Bioanalyse – Turkey
		Ticarcillin		TIC	
2	β-Lactam-β-lactamase inhibitor	Ticarcillin/clavulanic acid	75/10	TCC	
		Amoxicillin/Clavulanic acid	20/10	AMC	
3	Cephalosporines G^{III}, G^{IV}	Cefotaxime	30	CTX	
		Ceftriaxone	10	CRO	
		Ceftazidime	30	CAZ	
		Cefepime	30	FEP	
4	Fluoroquinolones	Ciprofloxacin	10	CIP	
		Levofloxacin	5	LEV	
		Oflaxacin	10	OFX	
5	Aminoglycosides	Gentamicin	10	CN	
		Amikacin	30	AK	
		Tobramycin	10	TOB	
		Streptomycin	10	S	
6	Monobactams	Aztreonam	30	ATM	
7	Carbapenems	Imipenem	10	IPM	
		Meropenem	10	MEM	

2.1.6 Bacterial Standered Strain

Table (2-7):The standard strain bacteria used in the current study

Strain name	Laboratory Identifier	Key Characteristics	Source
Pseudomonas aeruginosa	ATCC 27853	Sensitive for all antibiotics	The Ministry of Science and Technology

2.1.7 Primers

Table (2-8): The primers used in current study for genes detection (from Macrogen,Korea).

Type	Primer	Oligo sequence (5'-3')	Product size bp	Annealing temp °C	Reference
ESβL	***bla*$_{OXA10}$**	F-5`-TCA ACA AAT CGC CAG AGA AG-3` R-5`-TCCCACACCAGAAAAACCA-3`	277	55	AL-Kaisse *et al*.,2015
	***bla*$_{PER}$**	F- 5`-ATGAAT GTC ATT ATA AAAGC-3` R- 5`-AAT TTGGGC TTA GGG CAGAA-3`	926	56	Opus *et al* ., 2017
MBL	***bla*$_{IMP}$**	F-5` CTACCGCAGCAGAGTCTTTG3` R-5` AACCAGTTTTGCCTTACCAT3`	587	54	Doosti *et al* ., 2013
	***bla*$_{VIM}$**	F-5`-GTTTGGTCGCATATCGCAAC-3` R-5`-AATGCGCAGCACCAGGATAG-3`	384	55	Kazeminezhad *et al* ., 2017
	***bla*$_{NDM}$**	F5`-AATGGAATTGCCCAATATTATGC-3` R-5`-CGAAAGTCAGGCTGTGTTGC-3`	491	60	Bubonja-Sonje *et al*.,2015
AME	***aac(6')-Ib***	5`-TCCGTCACTCCATACATTGC-3` 5`-CGGTACCTTGCCTCTCAAAC-3`	304	60	Dakhl and Alwan,2015
	aac(3')-II	F-5`-CAAACGATGGGTGACGTATG-3` R-5`-CGTCGAACAGGTAGCACTGA-3`	212	60	Dakhl and Alwan,2015
	Ant(4')-IIb	F-5`-TCCTGTACCTGCGAATTGTG-3` R-5`-CTAGCGCCTCAACGGTATTC-3`	462	60	Dakhl and Alwan,2015
ERIC	**ERIC**	F-5`-ATGTAAGCTCCTGGGGATTCAC-3` R-5`-AAGTAAGTGACTGGGGTGAGCG-3`	variable	55	Khosravi *et al* ., 2016

Mex Efflux pumps	**MexB**	F-5`-ACTTCTTCAGCTTCAAGGAC-3` R-5`-GAGCATGAGGAACTTGTTG-3`	155	54	Poonsuk and Chuanchuen , 2014
	MexD	F-5`-CTACCCTGGTGAAACAGC-3` R-5`-AGCAGGTACATCACCATCA-3`	250	54	Poonsuk and Chuanchuen , 2014
	MexF	F-5`-CATCGAGATCTCCAACCT-3` R-5`-GTTCTCCACCACCACGAT-3`	350	54	Poonsuk and Chuanchuen , 2014
	MexY	F-5`-TCGCCCTATTCCTGCTG-3` R-5`-AGTTCGCTGGTGATGCC-3`	117	60	Fazeli *et al*.,2014
Integron ClassI	**IntI**	F-5-`GCATCCTCGGTTTTCTGG-3` R-5`-GGTGTGGCGGGCTTCGTG-3`	457	48	Kiddee *et al*.,2013
	In5`CS In3`CS	F-5`-GGCATCCAAGCAGCAAG-3` R-5`-AAGCAGACTTGACCTGA-3`	variable	55	Opus *et al* ., 2017
16Sr RNA	**16Sr RNA**	F-5`-AGAGTTTGATCCTGGCTCAG-3` R-5`-TACGGTTACCTTGTTACGACTT-3`	1500	60	Khosravi *et al* ., 2016

Table (2-9): The primers and Probes used for qRT-PCR detection of MDR genes (from Macrogen,Korea).

Primer	Oligo sequence (5'-3')	Product size bp	Annealing temp °C	Reference
***bla*$_{OXA10}$**	**F**-5-`GTCTTTCGAGTACGGCATTAG-3` **R**-5`-CATTGGTAGCGCAGGATTTA-3` **P**-FAM-ATTGACGGCTTCGGCAGAGAACTC - BHQ1*	129	55	This study
aac(6') - Ib	**F**-5`-GTTTCTTCTTCCCACCATCC-3` **R**- 5`-CCGTCACTCCATACATTGC-3` **P**-FAM-AATGGAGAGCCGATTGGGTATGCC -BHQ1*	100	55	This study
MexY	**F**-5` TCGCCCTATTCCTGCTG 3` **R**-5` AGTTCGCTGGTGATGCC- 3` **P**-FAM-AGGAGGGCCAGGATGTTC-BHQ1	112	60	This study
rpsl Refrence gene	**F**-5`- GCAAGCGCATGGTCGACAAGA-3` **R**-5`- CGCTGTGCTCTTGCAGGTTGTGA -3`	201	55	Arabestani *et al*.,2015

FAM=6-carboxyfluorescein ***BHQ1= black hole quencher**

2.2 Methods

2.2.1 Media Preparation and other Sterilization technique

A: Culture Media

All media (Nutrient agar, MacConkey agar, Brain heart infusion broth, Muller Hinton agar, Muller Hinton broth, Nutrient broth), were sterilized by autoclave at 121 °C and pressure 15 pound/ inch^2 for 15 minutes with adjusted pH at 7.0 ±0.3. They were prepared to the manufacturing company instructions manual. They were brought to boil on magnetic hot plate stirrer by magnetic bar at 100°C until constituents are being dissolved completely, and sterilized by autoclaving as mentioned. Then the media dispensed into sterile petri dishes or tubes as required and incubated at 37ºC for 24 hr to ensure sterility last stored at 4°C until use**.**

B: **Antibiotic Solutions**

The antibiotic solutions which can be damaged by high temperature were sterilized by filtrations through millipore filters paper (0.22 µm).

C: **Glass wares**

All glass wares were sterilized by dry heat in electric oven at 160 -180 °C for 2- 3 hours to avoid any contamination.

2.2.2 Laboratory Prepared Culture Media

2.2.2.1 Blood Agar Medium

These media were prepared according manufacturing company instructions(40gm/L) sterilized by autoclaving and cooled to 45-50°C. Then 5-10% of expired sterilized fresh human blood was added. These media were used for isolation; cultivating most of pathogenic bacteria and examining their ability to haemolys blood and showed type of hemolysis (Atlas, 2005).

2.2.2.2 Pseudomonas Agar Base

It was prepared according to manufacturing company instructions by dissolving 24.2 grams in 500 ml distilled water containing 5 ml glycerol. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and aseptically add sterile rehydrated contents of either CetriNix Supplement (FD029) or CFC Supplement (FD036) as desired. Mix well and pour into sterile Petri plates (Salfinger &Tortorello, 2015).

2.2.2.3 Triple sugar Iron agar slant (TSI).

For 500ml from this media 32.5gm is dissolved correctly on magnetic stirrer hot plate. Thereafter, it dispensed in test tubes and filled about 6-7ml for every tube. Then, it is sterilized by stem autoclave then cooled and sloped in an angle for slanted status of approximately 3.5-cm deep. The slant is approximately 2.5-cm which is acceptable. This media is a slant test tube that contains agar, a pH-sensitive dye (phenol red), 1% lactose, 1% sucrose, 0.1% glucose, as well as sodium thiosulfate and ferrous sulfate or ferrous ammonium sulfate for a successful differentiation gram negative bacteria (Hetsa *et al*. 2013).

2.2.3 Reagents , Stains and Solutions

2.2.3.1 Catalase reagent

A drop of catalase agent which is a ready commercial 3% hydrogen peroxide(H_2O_2) reagent was placed on a clean microscopic glass slide. This test is used for investigating the ability of bacteria to produce catalase enzyme which has causative degradation H_2O_2 releasing free O_2(Acharya, 2013) .

2.2.3.2 Oxidase reagent

The general name is Cytochrome C reagent in which the *N,N,N,N*- tetramethyl-p-phenylene diamine dihydrochloride ready reagent powder dissolution in 3 ml of

sterile distilled water or saline and immediately stored in dark container used to detect cytochrome C existing in specific gram negative bacteria (Hemraj *et al*., 2013).

2.2.3.3 Gram stain

Aready Kit consists of four containers :

a- Crystal violet solution, b- Lugol's iodine, c- Decolorization solvent and d- 0.5% standard safranine as counter-stain. This stain serves for differentiating between two types of gram bacteria (Barrow and Feltham, 1993).

2.2.3.4 Normal saline solution

85% saline was made by dissolving 0.85 gm of NaCl crystallization in beaker of 250ml and completed volume to 100 ml distilled water, then mixed via magnetic stirrers plate and bar until the salt is fully dissolved after that sterilized by autoclave and finally kept in closer bottle(Benson, 2001) .

2.2.3.5 Tris-borate EDTA Buffer (TBE)

TBE buffer (10 x) is comprised of Tris-base, glacial acetic acid and EDTA, so pH was adjusted to 8 and the volume was completed to 1000 ml distilled water. For preparing 1X TBE working solution, 100 ml of TBE (10 x) was added to 900 ml of D.W. It was preserved in room temperature till it would be being used in agarose gel electrophoresis (Stellwagen and Stellwagen, 2002).

2.2.3.6 Ethidium Bromide Solution (0.5 µg / ml)

Stock solution was prepared by dissolving 0.005 gm of ethidium bromide in 10 ml of D.W., and stored in dark reagent bottle.

2.2.3.7 Hydrochloric acid and NaOH 1M Solutions

According to dilution equation ($M1 \times V1 = M2 \times V2$) poured 8.26 mL of concentrated HCl(12.1 M and 36.46 g mol-1) to about 50 mL of D.W, completed up

to 100 mL. For 1M Sodium Hydroxide solution dissolved gently 4g of NaOH (F.W=40.00) pellets filled volume up to 100 ml of D.W, served to adjust pH of all solutions in this study (Flinn Scientific 2011).

2.2.3.8 EDTA Solution (0.5 M)

This solution was prepared by dissolving 190 mg of EDTA in 1 ml of D.W. After pH was adjusted to 8 by using Sodium Hydroxide solution NAOH, it was sterilized by autoclaving. EDTA and is used for the detection of metallo- beta lactamases producing isolates (Lee *et al*., 2003).

2.2.3.9 Preparation Stock Solution of Antibiotics

Antibiotics stock solution was prepared by dissolving 1g of antibiotic in 90 ml of D.W. and complete the volume to 100 ml. The reconstituted solution is a pale white solution with concentration of 10 mg/ml. Sterilize by filtration in milli pore in 0.22 mm diameter (CLSI, 2017). Serial dilution of antibiotics between (0.5-1024µg/ml).

2.2.3.10 Preparation solution of ZnO Nanoparticles(ZnONPs)

It was suspended with deionized distilled water by Ultra sonic homogenizer in room temperature for 30 min in concentration of the ZnO NPs was 10-30 nm , 81.38 g/mol MW, Purity 99.5% (Jamdagni *et al*.,2018). Serial dilution of ZnoNPs between (5200-5.07µg/ml).

2.2.4 Collection of Patient's samples and Culturing

A total of (326) clinical specimen were collected from the beginning of February 2018 to the end of August 2018 from patients in different hospitals of Baquba city (Baquba General Teaching Hospital, Educational Laboratories, Burns Specialist Center, Al- Batul teaching Hospital for Women and Children and Consultant of

Baquba General Teaching Hospital).The clinical specimens included are: wound swab(98), ear swab(50), burn swab(76) ,sputum(33) and urin (69).

Every Swabs and sputum,urin containers of 326 specimens were taken under sterile conditions and transferred, inoculated immediately on Blood agar and MacConckey agar at 37°C for 18 or 24 hour had been incubated, after that the good pure growth colonies cultuerd on specific selective media(Pseudomonas agar) and biochemical examines for more identification, diagnosis and characterization were carried out according to standard routine techniques.

2.2.5 Identification of clinical *Psudomonas aeruginosa* isolates

2.2.5.1 Morphological Examination

Primary diagnostic tests based on morphological characteristic of bacterial growth on Blood agar, Psuedomonas agar and MacConkey agar were studied including colony shape, colony texture, color and edges (Jawetz *et al*., 2016).

2.2.5.2 Microscopic Examination

Single pure colony of *P. aeruginosa* developed on nutrient agar for 18-24 hour at 37°C and the procedures had been done according to Christopher and Bruno,(2003).

2.2.5.3 Biochemical Tests

2.2.5.3.1 Catalase test (slide test)

Catalase production by *P. aeruginosa* which gives a positive test was done by picking the center of the 24 hour a good growth pure fresh culture colony with the help of sterile inoculating needle and mixed with a drop of 3% hydrogen peroxide(H_2O_2) reagent deposited on a clean glass slide. The appearance of immediate bubbles (gas liberation O_2) pointing to as positive for this test, while

bacteria gave no reaction catalase which is a negative result (Kasprowicz *et al*., 2018).

2.2.5.3.2 Oxidase test

Single isolated colony was transferred to a piece of filter paper by wooden stick, and then 2-3 drops of oxidase reagent were added to the filter paper. The change in color to dark purple within 20-30 seconds indicates a positive test. Oxidase-negative bacteria will not produce such changed color (Brown, 2005).

2.2.5.3.3 Growth on Pseudomonas agar

Pseudomonas agar is used for the selective isolation of *P. aeruginosa* from clinical specimens. It inhibits the growth of many microorganisms whilst allowing *P. aeruginosa* to develop typical luxuriant colonies.

2.2.5.3.4 Triple Sugar Iron slant (TSI) Test

This assay is used for the differentiation of microorganisms on the basis of dextrose, lactose, and sucrose fermentation with or without hydrogen sulfide production and gas releasing. A good single colony of *P. aeruginosa* isolate outgrowth on activation nutrient agar subculture picked up by disinfected needle, thereafter stubbed through the surface center of TSI slant agar(as in3.2.2.4) to the bottom of the tube and streaked the surface then incubated for 24 - 48 hr at 35-37°C after that the results were read (Perilla *et al.*, 2003).

2.2.5.4 Identification of *Pseudomonas aeruginosa* by Using VITEK 2 Compact System

The VITEK 2 Compact system is an automated microbiological system utilizing growth-based technology. It is characterized by accommodating the colorimetric reagent cards that are incubated and interpreted automatically, as shown in

Appendix(1). It is used to confirm the identification of both Gram negative and Gram -positive bacteria.

The VITEK-2 System is an identification system, which depends on the biochemical reactions between the bacterial isolates suspended in their solutions, and the media in the VITEK-2 Identification Cards, to identify the isolates. the bacterial isolates were inoculated on to MacConky agar plates and then incubated overnight at 37°C. A single colony was then taken and suspended into solution. the turbidity of the bacterial suspension was adjusted with VITEK Densichek (bioMerieux) to match the McFarland 0.5 standard in 0.45% sodium chloride. then the VITEK 2 ID-GN (Gram Negative) card and the bacterial suspension tubes were manually loaded into the VITEK-2 system.

Following steps on the software were done according to the manufacturer's instructions (BioMerieux, France) .

2.2.6 Preservation technique of bacterial isolates

All important bacterial isolates were maintenance performed followed on (Vandepitte *et al*., 2003**;** Murray *et al*., 2007) as follows:

2.2.6.1 Preservation for short-term

To store the isolate for one to three months in case of plate or slants, respectively, the procedure of Murray *et al*., (2007) was performed. Single pure colony of bacterial isolate is streaked on the nutrient agar culture plate and on the nutrient agar slants, and incubated at 37^{o}C for 24 hour, and then stored in the refrigerator at 4^{o}C.

2.2.6.2 preservation for Long-term

To maintain bacterial isolates for long period time (up three month), the process was carried out by culturing the isolated bacteria on media containing 20% glycerol at low temperature. The medium was prepared by adding 2ml of glycerol to 8ml of

brain heart infusion broth, dispensed in to small screw-capped bottle and sterilized by autoclaving. After cooling, the tubes was inoculated by one pure isolated colony and was incubated at 37 ºC for 24 hrs. The tubes were stored in deep freezing at -20°C (Vandepitte *et al*., 2003).

2.2.7 Antibiotics susceptibility test

The antibiotic-resistance profile of the planktonic pathogenic isolated organisms were evaluated by the typical Kirby-Bauer method according to Clinical and Laboratory Standards Institute(CLSI) guidelines27th(Patel *et al*., 2017) as follows:

1. Plates of Mueller-Hinton agar for the use in the Kirby- Bauer method for rapidly growing aerobic organisms were used. The medium in the plates was sterile and had a depth about 4 mm.
2. Inoculums preparation: pure colonies from overnight culture of tested isolates are transferred by cotton swab to a plain test tube containing 5.0 mL of normal saline to obtain suspension with a turbidity of 1.5×10^{8} CFU /mL and compare with MacFarland standards.
3. A sterile swab is dipped into the bacterial suspension, having any excess fluid expressed against the side of the tube.
4. Plates inoculation: The surface of a Mueller-Hinton agar plate was inoculated by bacterial isolate as follows: The whole surface of the plate was streaked with the swab, then the plate was rotated 45º angle and streaked the whole surface again; finally the plate is rotated another 90º and streaked once more.

5. Antibiotics disc warmed to room temperature and carefully placed onto the surface of above inoculated disc by using a sterile forceps. The disc was pressed gently on the agar surface to insure full contact, then the plates were incubated at 37ºC for 24 hour.
6. The diameter of each inhibition zone was measured with aid of a ruler in millimeter (mm). The isolate is interpreted as either susceptible, intermediate, or

resistant to a particular drug by comparison with stander strain and standards inhibition zone as shown in appendix (2).

2.2.8 Phenotypic Determination of β-Lactam-Resistance Mechanism

2.2.8.1 preparation of bacterial suspension

Inoculated test tubes containing 5 ml of normal saline by 150 µl of bacterial cultures growth in BHI agar for 18 hour, mixed well and comparing with McFarland standard solution.The bacterial suspension was prepared about (10^8) cell/ml concentration.

2.2.8.2 Detection of extended spectrum β-Lactamase production

The combined disk method was used to detect the ability of bacteria to produce ESBLs according to (Patel *et al*.,2017). And as the following:

1- Prepared the bacterial suspension as in (2.2.8.1) and separated 0.1 ml on petri dishes contain muller hinton agar and leave 10 min to let dry.
2- Affix a disk contain combination of Amoxicillin / Clavulanic acid (30 µg / Disk) in the center of inoculated plate. Then arranged the antibiotic disk of Aztreonam, Cefotaxime and Ceftazidime on distance of 3 cm of the disk in the center.
3- Incubated the plates in 37C for 24 hr.
4-The observation of incorporation of inhibition zone between the disk in the center and one or more of antibiotic disks around refers to positive result.

2.2.8.3 Detection of Metallo-β-Lactamase MBLs

The combination of antibiotic disk and EDTA method used to detect the ability of bacteria to produce MBLs (Bhalerao *et al* , 2010).

1- Prepared the bacterial suspension as in (2.2.8.1) and separated 0.1 ml on petri dishes contain muller hinton agar and leave 10 min to let dry.

2- Apply two disks of antibiotic Imipenem (10 mg) in the center of plate on a distance 3 cm between them .

3- Added 10 µl of EDTA reagent to one disc of Imipenem.

4- Incubated the plates in 37C for 24 hr.

5- After The observation of inhibition zone, the expansion of inhibition zone on 7 mm around the Imipenem disc with EDTA comparing with Imipenem disc alone refers to positive result.

2.2.9 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Determination

Minimum Inhibitory Concentration (MIC) is the lowest concentration of antibiotic that inhibits the visible growth of bacteria. The method of Stock and Ridg way, (1987) was applied for MIC determination by preparing a serial dilution of antibiotics between (0.5-1024µg/ml of Antibiotics). The experiment included the 3 control tubes: a tube containing sterile broth (Sterility control), a tube containing broth and bacterial isolate (Growth control), and a tube containing antibiotic and sterile broth. At last, 10 µl of bacterial suspension with turbidity equivalent to 0.5 MacFarland was added to the tubes contained different concentration of antibiotics, after 18-24 hr incubation at 37 C° the tubes were examined for growth. The MIC of antibiotic was taken as the lowest concentration that showed no growth. Additionally, the minimal bactericidal concentration (MBC) values were determined by sub - culturing the content of each tube without any growth, on the Mueller Hinton agar medium and looking for any bacterial growth.The MIC value was considered as the concentration with 99% bacterial growth inhibition and the MBC value was considered as the concentration with 100% of the inhibitory properties in comparison to the negative control (Alatoom *et.al*.2017).

2.2.10 Determination of Minimum Inhibitory Concentration (MIC) of ZnONPs against *Pseudomonas aeruginosa*

The minimal inhibitory concentration (MIC) value is the lowest concentration of ZnO NPs which prevents bacterial growths. Broth dilution method was used to determine the MIC. For this purpose, a stock of of ZnO NPs' suspension was prepared at a concentration of 1µg/µl (1 mg of NPs were mixed in 1 ml of distilled water) The Minimal Inhibitory Concentration (MIC) value is the lowest concentration of ZnONPs which prevents bacterial growths. Appropriate concentrations of ZnONPs samples were prepared according to their working manual, using Mueller Hinton broth in the range of 5.07-5200 µg/ml with serial two-fold dilutions of (5200,2600,1300,650,325,162.5,81.25,40.6,20.3,10.15,5.07µg/ml).A tube without nanoparticles was used as positive control. At last, 10 µl of bacterial suspension with turbidity equivalent to 0.5 MacFarland was added to the tubes contained different concentration of nanoparticles. After 24h from incubation at 37ºC, MIC values for each strain were determined by choosing the lowest concentrations in which no growth occurs. MIC was reported as the lowest concentration of the nanoparticles that inhibited visible bacterial growth. Additionally, the minimal bactericidal concentration (MBC) values were determined by sub -culturing the content of each tube without any growth, on the Mueller Hinton agar medium and looking for any bacterial growth. (Heshmati, *et.al*.2015).

2.2.11 Molecular Study of *P.aeruginosa* DNA

All molecular techniques in current study followed up the world scientific general biosafety conditions, also they carried out based on the manufacturer's kits recommendations and some time with simple slight modifications when critical requirement needed. Typically one milliliter and half of logarithmic-phase (A_{600} = 0.5) broth containing the range of ~10^6 - 10^8 (CUF/ml) used as bacterial pellet , performed by cultured overnight at 37°C in enriched broth media (Ma *et al*., 2006).

2.2.11.1 Total DNA extraction of *P.aeruginosa* isolates by Extraction Kit

Genomic DNA was isolated from bacterial growth according to the protocol of Wizard Genomic DNA Purification Kit, Promega as the following steps:

1- For pellet cells, 1ml of overnight culture for 2min at 13000 rpm. Supernatant then discarded.
2- 600µl from Nuclei Lysis Solution, was added and pipet gently for mixing.
3- All mixes were Incubate for 5 minutes at 80°C, then cooled to room temperature.
4- RNA lysis, 3µl of RNase solution, mixed, then incubated at 37°C for 15 minutes.
5- Protein Precipitation, 200µl of protein precipitation solution were added to cell lysate. Then mixed well by vertexing. Then incubated deep freeze (-30). After that, centrifuge at 13,000 rpm for 5 minutes.
6- Diluted DNA transfer to a clean tube containing 600µl of room temperature isopropanol. After mixing gently, centrifuge as in "Pellet Cells" above, and supernatant was decanted.
7- From room temperature 70% ethanol, 600µl were added then centrifuge for 2 minutes at 13,000 rpm.
8- Ethanol then aspirate and air-dried the pellet.
9- DNA pellet was rehydrated in 100µl of Rehydration Solution for 1 hour at 65°C. The tube contains the purified DNA. Store the DNA at -20°C.

2.2.11.2 Quantitation of extraction *P. aeruginosa* DNA

Quantus Fluorometer was used to detect the concentration of extracted DNA in order to detect the goodness of samples for downstream applications. For 1 µl of

DNA, 199 µl of diluted Quanty Flour Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected.

2.2.11.3 Estimation of DNA purity by Nanodrop

Nanodrop instrument was used to estimate DNA purity according to Sambrrok & Russell (2001), 1 µl of each DNA sample was applied in nanodrop for measuring the optical density (O.D) at wave length 260nm and 280nm. The purity was calculated according to formula:

DNA purity = O.D.260nm /O.D.280nm.

2.2.11.4 Molecular Determination of Antibiotic Resistance Genes By Polymerase Chain Reaction (PCR) Assay

2.2.11.4.1 Primer preparation

Forward and reverse primers which in lyophilization status were dissolved and diluted first in free nuclease D.S.D.W(amount according to recommended of manufactured company) to obtains 100 pico-mol/µl and this is considered as a stock solution, then it can be stored in deep freeze. This stock was diluted in free nuclease D.D.W to obtain nearly 10 pico-mol/µl and stored in deep freeze until used in PCR mixture.This technique accorded with all primers in this study, as listed in table (2-5).

2.2.11.4.2 Preparation PCR mixture

All of DNA that have been extracted from *Pseudomonas aeruginosa* isolates in current study went through PCR procedure in order to target the different drug resistance genes that are under study. Each PCR reaction had a final volume of 20 µl. In table (2-10) illustrated primers that are used in this study and their appropriate volume for PCR mixture. Promega master mix was used. The mixture composed from**,** PCR Master Mix (2x) solution which was melted at room temperature and mixed by vortex for homogenizing before being used. Primers solution were mixed well by vortex for homogenization before being used.

Table (2-10) Protocol of PCR reaction mixture volumes used in the current study.(Monoplex)

No.	Contents of reaction mixture	Stock concentration	Volume for a tube (µl)
1	Green master mix	2x	10
2	Forward Primer	10Pmol/µl	1
3	Reverse Primer	10Pmol/µl	1
4	DNA template	40ng	2
5	Nuclease free water	-	6
Total volume			20µl

***Final volume 20µl.**

2.2.11.4.3 Gradient PCR amplification procedure

A gradient PCR is often done to optimize PCR protocol and to figure out what annealing temperatures work best.PCR conditions were optimized by repeated changing annealing temperatures (from 48Cº to 60Cº according to primers) and number of cycles (30) according to current study till being fixed at the conditions listed in table (2-11a).This method offers significant time-savings and minimizes reagent use, relative to a standard PCR optimization protocol.

2.2.11.4.4 Thermal cycling conditions

All components of each PCR mixture were mixed together in Eppendorf tube by vortex before settings into thermocycler.The reaction was performed in a PCR thermal cycler apparatus, and after several trials, and according to the manufacture's guide. The following program in table (2.11a-2.11b) was adopted. Usually the process started with initial denaturation step (95ºC for 5 min) followed by repeated cycles which consists from denaturation step (ranged from 95ºC to 96ºC), annealing step (depends on the primers) then the extension step (mostly at 72 ºC) followed by final extension step (usually at 72°C).

Table (2-11a) Programs of PCR thermocycling conditions for detection of drug resistance genes

Genes Monoplex	Initial denaturation	Denaturation	Annealing	No of Cycle
bla$_{OXA10}$	95ºC / 5 min	95ºC/30 sec	55°C/30sec	30
bla$_{PER}$	95ºC / 5 min	95ºC/30 sec	56°C/30sec	30
bla$_{IMP}$	95ºC / 5 min	95ºC/30 sec	54°C/30sec	30
bla$_{VIM}$	95ºC / 5 min	95ºC/30 sec	55°C/30sec	30
bla$_{NDM}$	95ºC / 5 min	95ºC/30 sec	60°C/30sec	30
aac(6')-Ib	95ºC / 5 min	95ºC/30 sec	60°C/30sec	30
aac (3')-II	95ºC / 5 min	95ºC/30 sec	60°C/30sec	30
Ant(4')-IIb	95ºC / 5 min	95ºC/30 sec	60°C/30sec	30
ERIC	95ºC / 5 min	95ºC/ 1 min	55°C/30sec	30
MexY	95ºC / 5 min	95ºC/30 sec	60°C/30sec	30
IntI	95ºC / 5 min	95ºC/30 sec	48°C/30sec	30
In5`CS In3`CS	95ºC / 5 min	95ºC/ 1 min	55°C/30sec	30
16sRNA	95ºC / 5 min	95ºC/ 1 min	60°C/30sec	30

* Elongation in 72ºC\ 1 min and final extension 72°C\ 7 min for all genes.

Table (2-11b) Programs of PCR thermocycling conditions

PCR Program			
Steps	°C	m:s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	Depend on primer	00:30	
Extension	72	01:30	
Final extension	72	07:00	1
Hold	10	10:00	

2.2.11.4.5 Multiplex PCR For Detection of Efflux Pumps Genes

The primers were used were those as previously described by Poonsuk and Chuanchuen, (2014) table (2-8). They gave, respectively, amplicons of 155 bp (MexB-F and MexB-R), 250 bp (MexD -F and MexD R), 350 bp (MexF -F and MexF- R). Each PCR reaction had a final volume of 20 µl. In table (2-12) illustrated primers that are used in this study and their appropriate volume for PCR mixture.

Table (2-12) Volumes of PCR mixture were used in current study.(Triplex)

No.	Contents of reaction Mixture	Stock concentration	Volume for a tube (µl)
1	Green master mix	2x	10
2	Forward Primer MexB	10Pmol/µl	1
3	Reverse Primer MexB	10Pmol/µl	1
4	Forward primer MexD	10Pmol/µl	1
5	Reverse primer MexD	10Pmol/µl	1
6	Forward primer MexF	10Pmol/µl	1
7	Reverse primer MexF	10Pmol/µl	1
8	DNA template	40ng	2
9	Nuclease free water	-	2
Total volume			20µl

Amplification was carried out with the following thermal cycling conditions: 5 min at 95°C and 32 cycles of amplification consisting of 30 s at 95°C, 30 s at 54°C, and 50 s at 72°C, with 7 min at 72°C for the final extension.

2.2.11.4.6 Agarose Gel Electrophoresis

According to Michael and Sambrook (2012) PCR amplicons were analyzed by using agarose gel electrophoresis and several steps were followed as mentioned below:

1- Agarose (1%) was prepared by dissolve 1gm in 100 ml of 1X TBE, then it was melted by heating with stirring. The agarose was left to cool to 60°C, then 1 µl ethidium bromide was added (final concentration 0.5 µg/mL).
2- The agarose was poured carefully into the tapped tray to avoid formation of bubbles .
3- A comb was placed near one edge of electrophoresis tray. It was left at room temperature for 30 min to solidify and became obscure. then the comb and the tape were gently removed.
4- TBE (1X) electrophoresis buffer was poured into gel tank for filling tank about 3-5 mm above the gel and making sure that all the gel is completely covered. and the tray was placed horizontally in electrophoresis tank.
5- The PCR master mix already containing loading buffer so the amplified PCR products were loaded directly in the wells.
6- Five micro liters of the DNA ladder (100bp) were loaded in single lane which served as marker during the electrophoresis process. DNA moves from Cathode to plus Anode poles. The ethidium bromide stained bands in gel were visualized using Gel imaging system.
7- The power supply was set at 100 V/mAmp for 75min. till the trapping dye reached the end of the gel.
8- The gel was exposed to UV Transilluminator (320 nm/360w) then photographed by digital camera.

2.2.12 Molecular study of *P. aeruginosa* RNA

2.2.12.1 Total RNA extraction

RNA was isolated from sample according to the protocol of TRIzol™ Reagent as the following steps:

1-Sample lysis

Cells grown in suspension: For pellet calls, 1.4 ml of cells culture was precipitate by centrifugation for 2 min at 13000 rpm, supernatant then discarded and 0.75 mL of TRIzol™ Reagent was added to pellet.

The lysate was homogenized by pipetting up and down several times.

2- Three phases separation

- For each tube, 0.2 mL of chloroform was added to the lysate, then the tube cap secured.
- All mixes were Incubate for 2–3 minutes then centrifuge for 10 minutes at 12,000 rpm, the mixture was separated into a lower organic phase, interphase, and a colorless upper aqueous phase.
- The aqueous phase containing the RNA was transferred to a new tube.

3- RNA precipitation

- 0.5 mL of isopropanol was added to the aqueous phase and incubated for 10 minutes then centrifuge for 10 minutes at 12,000 rpm.
- Total RNA was precipitate formed a white gel-like pellet at the bottom of the tube.Supernatant then discarded.

4- RNA washing

- For each tube, 0.5mL of 70% ethanol was added and vortex briefly then centrifuge for 5 minutes at 10000 rpm.

- Ethanol then aspirated and air-dried the pellet.

5- RNA solubility

- Pellet was rehydrated in 20-50 µl of Nuclease Free Water then incubated in a water bath or heat block set at 55–60°C for 10–15 minutes.

2.2.12.2 Quantitation of RNA

Quantus Florometer was used to detect the concentration of extracted RNA or cDNA in order to detect the goodness of samples for downstream applications. For 1 µl of RNA or cDNA, 199 µl of diluted QuantyFlour Dye was mixed. After 5min incubation at room temperature in dark place, RNA concentration values were detected. Nanodrop instrument was used to estimate RNA purity according to Sambrrok & Russell (2001).

2.2.12.3 Primer and Probe preparation

Specific primers and probes were designed in current study for three genes used in gene expression *bla$_{OXA10}$, aac(6)'Ib and MexY* by using IDT primer and probe design In silico program(Primer Quest Tool) Appendix (8). Forward and reverse primers and probes which in lyophilization status were prepared as same of procedure in step(2.2.11.3.1).

2.2.12.4 Confirm phenotypic Drug Resistance by quantitativeRT-PCR of *bla$_{OXA10}$, aac(6)'Ib and MexY* gene expression

To confirm and select the highest and lowest drug resistance isolates through of gene expression which is determined and based on comparing the difference values of mean C_T of *bla$_{OXA10}$, aac*(6)'Ib and MexY genes and confirmed and normalized them by $2^{-\text{mean } \Delta C_T}$, where the ΔC_T; is the difference in C_T threshold cycle between the target and reference gene obtained from quantitative real time-PCR(qRT-PCR) for *bla$_{OXA10}$, aac*(6)'Ib and MexY (target genes) and *rps*L(reference gene) each

isolate, so $\Delta C_T = C_{T\ gene} - C_{T\ reference\ gene}$, where C_T; is threshold cycle value for the amplified gene (Rao *et al.*,2013). The specific primers for qRT-PCR and gene expression were Designed for *bla$_{OXA10}$, aac(6)'Ib and MexY* genes in this study and listed in table(2-9).

The standard protocol was applied on extracted RNA with TRIzol™ qPCR Master Supermix TaqMan (1-Step RT-qPCR System) used according to manufacturer's instructions and applies to a single reaction where only template, primers,probes and water need to be added to the qPCR Master (TaqMan). All reagents should be thawed on ice, gently mixed and briefly centrifuged before use.

1. Thaw reagents at room temperature. Mix thoroughly and then place on ice immediately after thawing.

2. Assemble reaction tubes on ice or sub-zero freeze plate, whenever possible to avoid premature, nonspecific polymerase activity.

3. The following table shows recommended component volumes:

Table(2-13):Ingredients and their volumes for qRT-PCR technique of the *bla$_{OXA10}$* , *aac*(6)'Ib and *MexY* genes.

No.	Contents of reaction mixture	Stock concentration	Volume for a tube (µl)
1	qPCR Master Mix	2x	5
2	RT mix	50x	0.25
3	MgCl2		0.25
4	Forward primer	10Pmol/µl	0.5
5	Reverse primer	10Pmol/µl	0.5
6	Probe	10Pmol/µl	0.5
7	Nuclease Free Water		1.5
8	RNA	40ng/µl	1.5
Total volume			10µl

***Final volume 10µl.**

Table(2-14):Ingredients and their volumes for qRT-PCR technique of *rps*L gene.

No.	Contents of reaction mixture	Stock concentration	Volume for a tube (µl)
1	qPCR Master Mix	2x	5
2	RT mix	50x	0.25
3	MgCl2		0.25
4	Forward primer	10Pmol/µl	0.5
5	Reverse primer	10Pmol/µl	0.5
6	Nuclease Free Water		2
7	RNA	40ng/µl	1.5
Total volume			10µl

***Final volume 10µl.**

4- Ensure reactions are mixed thoroughly by pipetting or gentle vortexing followed by a brief spin in a microcentrifuge.

5- Transfer tubes into a Real-time PCR instrument and the program runs as the following table (Dumas *et al*.,2006).

Table(2-15):qRT-PCR programed detection of the *bla*$_{OXA10}$, *aac(6)'Ib* , *MexY* and *rps*L genes

Step	Temp ºC	Time	Cycle
RT. Enzyme Activation	37	15min.	1
Initial Denaturation	95	10min.	
Denaturation	95	30 sec.	40
Annealing	55	30 sec.	
Extension	72	30 sec.	
Final extension	72	07:00	1
Hold	10	10:00	

Quantitative real-time PCR and gene expression were performed using the Mic qPCR Cycler -RT-PCR Detection System (Bio Molecular System, Australia).

Based on the result of mean ΔC_T and $2^{-\Delta CT}$ with the least and highest values respectively, each tested isolate was selected for studying Ceftazidime and Gentamicin impact on changing in gene expression of bla_{OXA10} , *aac(6)'Ib and MexY* genes as in treatise of(Galil *et al*.,2013).

2.2.12.5 Effect ZnONPs on gene expression of Drug Resistance bla_{OXA10} , *aac(6)'Ib and MexY* genes

Mechanistically investigated the effectiveness exposed of *P. aeruginosa* isolates to ZnO NPs with MIC and sub-MIC on in vitro drug resistance $bla_{OXA10,}$ *aac(6)'Ib and* MexY genes expression, as the same procedure mentioned in (2.2.9), used for bacterial growth and isolates exposure to zinc oxide NPs according to(Gupta *et al*., 2014;Yousry *et al*., 2016), then the protocol in table(2.13) was accomplished for qRT-PCR and change "increase or decrease" in expression of bla_{OXA10} , *aac(6)'Ib and MexY* genes relatively to the normalize, also the *rps*L gene was used as both a reference for data normalization and housekeeping gene internal control to ensure data reliability(Yu *et al*., 2016).

Data analysis was performed using the $2^{-\Delta\Delta C_T}$ method, where $\Delta\Delta CT$ = ΔCT(treated isolate with ZnO NPs) - ΔCT(untreated isolate with ZnO NPs), ΔCT= CT(target gene) - CT(*rps*L), and C_T is the threshold cycle value for the amplified gene(Schmittgen and Livak, 2008; Xie *et al*.,2011;Yang and Alvarez, 2015).

2.2.13 Sequencing and Blasting

Sequencing was performed for 7 genes of the 2 isolates. Uniplex PCR products of bla_{OXA10} , bla_{VIM} , *aac(6)'Ib* , *MexY,MexD* and *MexF* genes samples of *Pseudomonas aeruginosa* isolates were stored at -20C°, then the nucleotides sequence of gene carried out by sending the samples and primer to Macrogen Corporation – Korea. After the results were received, the homology search was conducted using Basic

Local Aligmment Search Tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online .

2.2.13.1 DNA Sequencing of PCR amplicons 16S rRNA

The resolved 1500 bp PCR amplicons were commercially sequenced from both directions, forward and reverse termini, according to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed DNA sequences of local samples with the retrieved DNA sequences of *P. aeruginosa*, the virtual positions and other details of the retrieved PCR fragments were identified.

Table (2-16) The specific primers' pair selected to amplify three loci within *Pseudomonas aeruginosa* genomic DNA sequences. The symbols (+) and (-) refer to the orientation of the positive and negative strand, respectively. The bold letters refer to the start and end of the amplicon fragment.

Primer	Sequence (5′-3′)	Amplicon size	GenBank Accession Number	Annealing temperature
27-F	AGAGTTTGATCCTGGCTCAG	1500 bp	CP012901.1 (**223007**–223026) +	60°C
1492-R	TACGGTTACCTTGTTACGACTT		(224485–**224506**) -	

2.2.13.2 Interpretation of sequencing data

The sequencing results of the PCR products of the targeted bacterial sample were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were numbered in PCR amplicons as well as in its corresponding position within the referring genome.

2.2.13.3 Comprehensive phylogenetic tree construction

A specific comprehensive bacterial tree was constructed in this study according to the protocol described by Al-Shuhaib *et al.* (2019). The observed bacterial variants were compared with their neighbor homologous reference sequences using NCBI-BLASTn server (Zhang *et al*. 2000). Then, a full inclusive tree, including the observed variant, was built by neighbor-joining method and visualized as polar cladogram using Figtree tool (http://tree.bio.ed.ac.uk/software/figtree/). The bacterial sequences of each classified phylogenetic species-group in the comprehensive tree were colored appropriately.

2.2.14 Statistical Analysis

Statistical analysis was performed with Graph Pad Prism version 6 software, percentages were used for the comparison between samples of the study. Data analysis was done using Chi-square for the comparison of categorical data.

3. Results and Discussion

3.1 Isolation of *Psudomonas aeruginosa*

In this study, The results revealed that a total number of 81(27.6%) isolates of *Pseudomonas aeruginosa* were obtained from 326 clinical specimens in which there were 293 specimens that had been given positive growth (33 specimens no growth) initially diagnosed in hospitals from various body sites of infections from both male and female, different ages, diverse local regions and together urban and rustic habitat. The isolates were collected during the study period from initial February /2018 finished in the end of August /2018. These were collected from governmental hospitals in Baquba / Diyala.

3.2 Identification of *Psudomonas aeruginosa*

Total of positively grew was 293 that had been isolated from the clinical specimens of five different infectious sources;(burns, wounds, urin,respiratory sputum, and ear swabs (otitis media)). All samples were subjected to the common microbiological detection methods involved diversified of enrichment, differential and selective media, colony morphological and cells microscopically by gram dye, and assorted of biochemical tests.

The results of all traditional bacteriology tests had showed that of 293 clinical specimens gave positive growth which was cultured and grown on blood then MacConky agar(Ghaima, 2016) . Besides, there were just 81(27.6%) isolates which gave obvious indicators to identify the target bacteria, therefore, all efforts by these tests had provided a primary diagnosis in the beginning as *Pseudomonas aeruginosa*, the results of media growth and traditional biochemical tests are listed in table(3.1).

Table (3-1): Results of traditional microbiological detection methods of *Pseudomonas aeruginosa*.

No.	Media and test	Result
1	Growth on MacConkey agar	+
2	Lactose fermentation	-
3	Catalase	+
4	Oxidase	+
5	Gram stain	-
6	Growth on Pseudomonas agar	+
7	Blue- greenish(Pyocynine) color	+/-
8	Growth at 42-44ºC overnight	+
9	growth at 4ºC overnight	+
10	Triple Sugar Iron slant	Alk / Alk, No H_2S, No gaseous

(+) Positive result and (-) Negative result

All 81 isolates were able to grow on MacConkey agar media as a selective(inhibit most Gram positive bacteria and allow to grow Gram negative bacteria) and so a differential (between lactose ferment gram negative and non-lactose ferment gram negative) in which *P. aeruginosa* non-lactose ferment grew with pale yellowish color. In addition, the growth on Pseudomonas agar and blue greenish color produced pyocyanine on overnight are a specific selective media designed to select *P. aeruginosa* from other *Pseudomonas* species. The production of some pigments on Nutrient or Muller-Hington agar was especially associated with *P. aeruginosa* as in figure (3-1). These results agreed with what AL-Shamaa *et al*. (2016) found for identifying *P. aeruginosa* and other bacteria isolated from burns and wounds Iraqi specimens . These pigments besides their role in *P. aeruginosa* pathogenicity as virulence factors, they are playing as a character of pigmentation which remains significant factors among the diagnostic traits

in the genus of *Pseudomonas*; therefore; they are used by Jayaseelan *et al*. (2014); Novik *et al*. (2015) for diagnosing this bacteria.

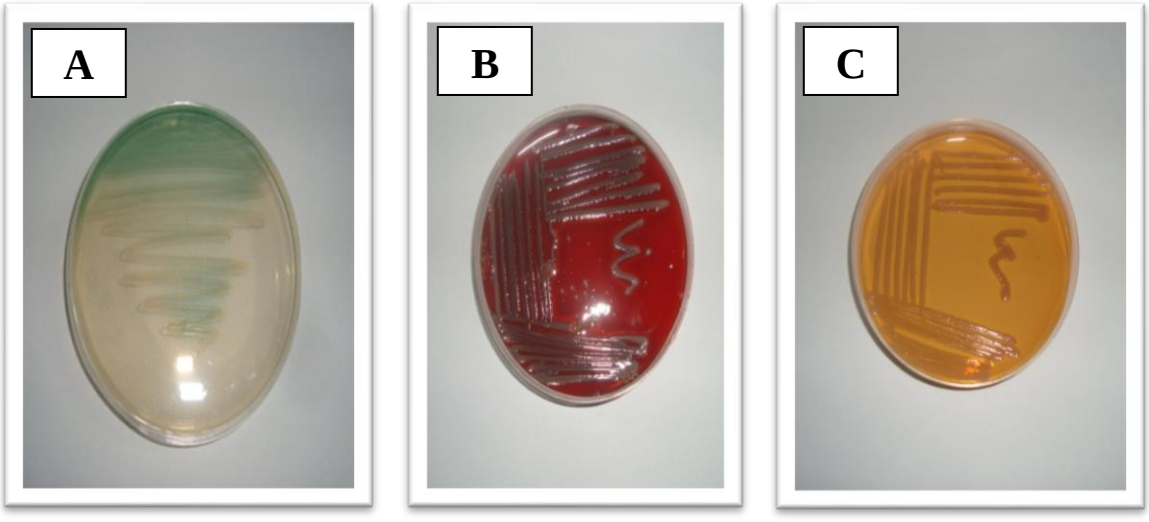


Figure (3-1): *Pseudomonas aeruginosa* Cultured (37°C for 24 hrs **) on:**

(A) Pseudomonas agar (B) Blood agar (C) MacConky agar

All 81 isolates subjected to oxidase test, catalase test, triple sugar iron (TSI) test and other biochemical tests. The violet color (+ result) had appeared with isolates which refer to activity of cytochrome C, and bubble formation (+ result) mean capability to degrade H_2O_2 by catalase. The unchanged color (stayed pink) of slant and bottom, no black color media appeared and un cracking in structure of agar template meant to non-ferment any sugar, no H_2S production, and no gas release respectively. These results were analogical with what was recommended by Gillespie and Hawkey, (2006) for detecting *P. aeruginosa* species.

Microscopically examination was achieved for target isolates using differentiated dye by Gram stain, so the 81 isolates mostly had been tinted with pink color referred to negative gram, and appeared straight or slightly rods shapes, this was compatible with what referred by The Standards Unit Microbiology Services,(2015) to identify *Pseudomonas* species and other non-glucose fermenters.

VITEK 2 system used the identification card for Gram negative isolates (IDGNB).All 81 clinical isolates were identified as *P.aeruginosa* by VITEK 2 system using the identification card for Gram negative strains (IDGNB); The example of report resulted by this system for identification these bacteria was shown in an Appendix (2). This system used in many previous studies and gave good results for identification and confirmation the biochemical tests. The anti-biograms of *P. aerugenosa* isolates could be determined by this automized system (Abdalhamid *et al*., 2014; Guckan *et al.*, 2015). The findings of Funke *et al.* (1998) indicated that the VITEK 2 system was shown to identify correctly 84.7% of selected species of members of the family Enterobacteriaceae and nonenteric bacilli including 64 different tests within 3hrs. Zbinden *et al*. (2007) reported that the accurate identification of *P. aeruginosa* by using VITEK 2 system. The results of current study were similar with the study of Jaafar *et al*. (2014) in Baghdad and AL-Mayyahi (2018) in Wasit.

3.3 Distribution of *Pseudomonus aeruginosa* according to the source of Infection

Out of 326 clinical samples of wound, urine, burns, ear, and sputum, 81 isolates were positive for *P. aeruginosa*. These positive isolates were obtained in high percentage; 29.62% (n=24) from wound samples; while the percentage of burn samples were 25.92% (n=21), urine samples constituted 20.98% (n=17), ear samples 16.04% (n=13) and the low percentage was obtained from sputum samples which achieved 7.40% (n=6). Number and percentage of *P.aeruginosa* isolates according to samples source are shown in Table (3.2). When comparing the results of this study with other Iraqi research, it was noted that there was a difference in the percentage of *P. aeruginosa* isolates Jabbar (2015), and Najim (2016) found that the percentage of *P. aeruginosa* only from Burn's patients 60.0% and 41.% respectively.

Table (3-2): Numbers and Percentages of *Pseudomonas aeruginosa* among Different Clinical Sample.

Type of infection (specimens)	No. of samples &(%)	No. of *P. aeruginosa* &(%)	Percentage of isolates to samples
Wound Infection (Swab)	98 (30.06%)	24/81(29.62%)	24/98(24.48%)
Burn Infection (Swab)	76 (23.31%)	21/81 (25.92%)	21/76(27.63%)
Ear Infection (Swab)	50 (15.33%)	13/81 (16.04%)	13/50(26%)
Urinary Tract Infection (Urine)	69 (21.16%)	17/81 (20.98%)	17/69(24.63%)
Respiratory Tract Infection (Sputum)	33 (10.12%)	6/81 (7.40%)	6/33(18.18%)
Total	326 (100%)	81/81 (100%)	81/326(27.6%)

The study varies in proportions with another Iraqi investigation by Neamah (2017) that found the total of 50 different human samples isolated from Al-Diwanyia hospital, the number and percentage of *P. aeruginosa* isolates were highest from burns 14/20(70%), followed by otitis 11/16(68%) and wound 6/14(42%), while 60 isolates from Baghdad hospitals were collected by Abd.(2018) that achieved 17(34%) from burns,15(30%) ear infection, 11(22%) wound and 2 (4%) from urine and sputum. Latif (2016) found that the percentage of isolates obtained from burns was (32.5 %), while the percentage of isolates obtained from wound was (23.2 %).*P. aeruginosa* is currently considered the main agent of acquiring ventilator-associated pneumonia (VAP) and in the intensive care units(ICU), causing an average of approximately 50% of VAP cases(Lima *et al*., 2017).

These rations vary from one study to another. The variation of the presence of *P. aeruginosa*, among the infected isolates, may attributed to major factors including the

differences in the type of samples, isolating source, the sampling time, method, season of sampling, number of collected samples, , the geographical area that the samples were obtained from and other conditions that differ among studies of this type.

3.4 Antibiogram Pattern of *Pseudomonas aeruginosa*

To estimate potential resistance of *P. aeruginosa* isolates against 18 items of antibiotics from different classes, all 81 isolates had been subjected to antibiogram test according to Clinical and Laboratory Standards Institute(CLSI-2017) guidelines(Performance Standards for Antimicrobial Susceptibility Testing; 27th Informational Supplement) by(Patel *et al*., 2017), and this assay could be preferably achieved by widespread Kirby-Bauer disk diffusion technique(Bauer *et al*., 1966) .

When the 81 *P. aeruginosa* isolates were subjected to the antibiotic susceptibility testing against 18 antipseudomonal agents, the results in table (3.3) showed that isolate varied in their resistance and sensitivity to the antibiotics. It was found that 74.07% and 85.18% of the isolates were resistant to piperacillin and ticarcillin, respectively. For β-lactam/β -lactamase inhibitor combination agents, 93.82% and 71.60% of isolates appeared resistance to Amoxicillin/Clavulanic acid and ticarcillin-clavulanic acid, respectively. The rates of resistance to the third generation cephalosporins were as follows: ceftriaxone 87.65% , cefotaxime 85.18% and ceftazidime 75.30%. Additionally, 80.24% of the isolates exhibited resistance to the fourth generation cephalosporin, cefepime.

The overall resistance rate to aminoglycoside antibiotics was high, with a maximum resistance aginst streptomycin 90.12%, followed by gentamicin 85.18% ,tobramycin 65.43%, while the moderate resistance rate was observed for amikacin (56.79%). According to the results of the fluoroquinolones susceptibility testing, 51.85% , 59.25% and 69.13% of the isolates were resistant to ciprofloxacin, levofloxacin and oflaxacin, respectively. Resistance to monobactam was moderate when 50.61% of isolates being resistant to aztreonam, while the lowest resistance was observed for

carbapenems. Resistance to imipenem and meropenem were 11.11% and 23.45%, respectively.

Table (3-3): Antibiogram pattern of *Pseudomonas aeruginosa* isolates toward antipseudomonal agents (n= 81).

Antibiotic		Resistant isolates No. & %	Intermediate isolates No. & %	Sensitive isolates No. & %	*p-value*[a]
Class	**Type**				
1	Pipracilin	60(74.07%)	5(6.17%)	16(19.75%)	0.008
	Ticarcillin	69(85.18%)	3(3. 70%)	9(11.11%)	0.005
2	Ticarcillin/clavulanic acid	58(71.60%)	6(7.40%)	17(20.98%)	0.009
	Amoxicillin/Clavulanic acid	76(93.82%)	1(1. 23%)	4(4.93%)	0.001
3	Cefotaxime	69(85.18%)	4(4.93%)	8(9.87%)	0.005
	Ceftriaxone	71(87.65%)	5(6.17%)	5(6.17%)	0.004
	Ceftazidime	61(75.30%)	2(2.46%)	18(22.22%)	0.008
	Cefepime	65(80.24%)	4(4.93%)	12(14.81%)	0.017
4	Ciprofloxacin	55(67.90%)	-	26(32.09%)	0.073
	Levofloxacin	59(72.83%)	-	22(27.16%)	0.056
	Oflaxacin	56(69.13%)	7(8.64%)	18(22.22%)	0.042
5	Gentamicin	69(85.18%)	2(2.46%)	10(12.34%)	0.008
	Amikacin	46(56.79%)	8(9.87%)	27(33.33%)	0.051
	Tobramycin	53(65.43%)	11(13.58%)	17(20.98%)	0.038
	Streptomycin	73(90.12%)	2(2.46%)	6(7.40%)	0.007
6	Aztreonam	41(50.61%)	10 (12.34%)	30(37.03%)	0.064
7	Imipenem	9(11.11%)	7(8.64%)	65(80.24%)	0.023
	Meropenem	19(23.45%)	12 (14.81%)	50(61.72%)	0.038

a: ***P-value*** was calculated using the Chi-square test in terms of the R, I & S group.

†class(1):):[Penicillins-Ureidopenicillin, Penicillins-Carboxypenicillin].(2): β-Lactam-β-lactamase inhibitor combinations Sub-class(3) : [Cephalosporines G^{III}, G^{IV}] class (4): [Fluoroquinolones]. class (5): [Aminoglycosides]. Sub-class(6):[Monobactams] Sub-clas (7): [Carbapenems]. (Patel *et al*., 2017). Classes=class and sub-class.

During the study period, 74.07% of the isolates were found to be resistant to piperacillin, this result is agreed with the value reported by Hussein *et al.* (2018) for

piperacillin 67.96% in Wasit/Iraq. While in previous reports published from Baghdad hospitals by(Al-Kaisse *etal*.,2015) reports of the susceptibility of *P. aeruginosa* to piperacillin was 80% and study by (Azeez and Bakr , 2019) in Irbil/Iraq revealed that 100% of *P. aeruginosa* resistant to piperacillin .

Pseudomonas aeruginosa isolates across countries are increasingly resistant to a higher number of antimicrobial agents. Ali (2016) described that among 60 isolates of *P. aeruginosa*, 30% resist to 14 different antibacterial agents. A study done by Al-Saffar and Jarallah (2019) showed that all 90% of clinical isolates of *P. aeruginosa* in Babylon, Iraq were resistant to Ceftazidime, Cephalexin and Ticarcilline.

During nineties, third generation cephalosporin was the empirical antibiotic choice for patients infected with Gram-negative bacteria at Diyala province. However, rising trend of resistance against this group of antibiotics was observed among *Enterobacteriaceae* and *P. aeruginosa* (Al-Shara, 2013). Based on present results, the examined third generation cephalosporins; ceftriaxone, cefotaxime and ceftazidime effective against isolates tested were, 87.65%, 85.18% and 75% identified as resistance, respectively. This result agreed with Al-Saffar and Jarallah (2019) reports that percentage of resistance to ceftriaxone, cefotaxime and ceftazidime were 90%,87%,85% respectively. While, study by Tawfeeq *et al*. (2017) in Tikrit/Iraq revealed that resistance percentage to Cefotaxim (60.34%). As well, this reduced susceptibility may be related to the more extensive use of this group of antibiotics in Diyala hospitals. On the other hand, additional resistance mechanisms especially production of ESBLs and other enzymes may contribute to third generation cephalosporins resistance (AL- Rubaye *et al*., 2015).

Extended spectrum fourth generation (cefepime) displayed an increased stability against enzymatic degradation by β-lactamases (AmpC -lactamases in particular), and an enhanced ability to penetrate the porins in the outer membrane of Gram negative bacteria (Bonomo and Szabo, 2006). Cefepime is the commonest fourth generation

antibiotics in hospital protocols. In this study, there was a relatively resistance rate to cefepime (80.24%), which was high as compared to other local study, 53.40% (Hussein *et al.* 2018) and low as compared to other local study, 100% (Al-Kaisse *etal*.,2015).

The percentage of fluoroquinolone-resistant isolates was 67.90% and 72.83% of isolates resistant to ciprofloxacin and Levofloxacin, respectively identified in this study is considerably lower than that reported in study conducted in Iraq wasit Hospitals, in which resistance were 83.50% for ciprofloxacin (Hussein *et al.,* 2018). Results conducted in current study agreement with Al-Wasity, (2018) who reported that *P.aeruginosa* isolates developed resistance to different antibiotic classes, including flouroquinolones in high resistance rates were 64.5% and 74.2% of isolates resistant to ciprofloxacin and Levofloxacin, respectively.

The results of the current study are nearly in agreement with the studies conducted by Hussein and Shamkhi (2018)in wasit hospitals, who reported that their *P. aeruginosa* clinical isolates were resistant to gentamicin (91.26%). Burgh *et al.* (2018) demonstrated that *P. aeruginosa* isolates in Kurdistan, Iraq were gentamicin, tobramycin resistant; however at a resistant rate of (88% and 70%), respectively.

Results would support some data found in local study done by Al-Saray (2016) who reported high resistance rate toward cefotaxime represented by (91.3%) of *P.aeruginosa* isolates, followed by carbencillin (76.19%), ciprofloxacin (74.62%) and (71.43%) to gentamycin, whereas (43.48%) of I solates were resistance to ceftazidime.

Multi-drug resistant health care-associated *P.aeruginosa* infections can be fatal for patients, that estimated 51,000 infections occur in the United States each year, more than 6,000 (13%) of these are MDR with severely 400 deaths/ year attributed to these infections (CDC, 2018).

Limited antimicrobial effectiveness against *P.aeruginosa* is due to the pathogen's broad range of intrinsic and adaptive antimicrobial resistance mechanisms. Chromosomally, *P.aeruginosa* encodes an AmpC betalactamase (granting resistance to

many penicillins and cephalosporins), has an OprD outer membrane porin which can be variably expressed (loss of which confers resistance to carbapenems); these are all inducible and regulation is dependent upon the environment encountered by the organism (Moore *et al*., 2016).

Based on the results, imipenem showed better activity (80.24%) than meropenem (61.72%) in study period. Shaaban *et al*.(2017) mentioned that 16/90 of *P.aeruginosa* clinical isolates, showed high levels of resistance to meropenem and imipenem resulted from the prevalence of metallo-beta-lactamase encoded by Verona integron (VIM),which confirmed by PCR analysis of carbapenemases.

P. aeruginosa is a versatile opportunistic pathogen. It is the predominant cause of wound infection in pre-antibiotic and persists as critical pathogen, strongly studies as a significant cause of nosocomial infections. In recent years, a marked increase in the number of hospitals acquired infections due to multidrug resistance has been reported from many countries (Ekrem and Rokan, 2014).

Different antibiotics are commonly used for the treatment of *P. aeruginosa* infections, such as aminoglycosides, cephalosporins- mostly generation III and IV beta-lactamas, penemes and quinolones, also,Carbapenems(sub-class of penemes) are potent β-lactam antibiotics against Metallo- βeta lactemase (MBLs) producing and multidrug resistance *P. aeruginosa* , there have been many recent reports that clinical isolates of *P. aeruginosa* are becoming resistant to carbapenems in many countries(Aghamiri *et al*., 2014).

MBLs producing *P. aeruginosa* is a serious intimidation in hospital locations, especially in burn units. These strains can create significant problem in treatment and spread of resistance among other bacteria(Mirbagheri *et al*., 2015). Resistance to carbapenems via acquirement of MBLs genes among *P. aeruginosa* strains have increased rapidly in Asia, Europe, and South America. This has led to a drastic change

in the pattern of antibiotics usage against multidrug resistant *P. aeruginosa*(Aghamiri *et al*., 2014).

One of the reasons for the development and increased resistance to antimicrobial classes like the antipseudomonal penicillins, penicillin–inhibitor combinations, cephalosporins, carbapenems and monobactams in *P. aeruginosa* mostly results from overexpression of its naturally occurring AmpC-type cephalosporinase, because the isolates with the wild-type AmpC produce only low basal levels of the enzyme and are susceptible to these classes(Qing *et al*., 2014) .

3.5 Multi-drug Resistance of *P*seudomonas *aeruginosa* Isolates

The incidence of Multi-drug resistance (MDR), extensive drug resistance (XDR) and pan-drug resistant (PDR) was investigated among isolates of 81 *P. aeruginosa*. MDR isolates were defined as isolates demonstrating resistance to antimicrobials from at least three of the seven-antipseudomonal classes of antimicrobial drugs tested in this study: aminoglycosides, antipseudomonal penicillins, carbapenems, cephalosporins, β-lactam/ β-lactamase inhibitor combination, monobactams and quinolones. While, the definition of extensive drug resistance (XDR) is an isolate that is resistant to all but one or two classes. PDR isolates are non-susceptible to all seven antimicrobial categories tested (Magiorakos,2012).

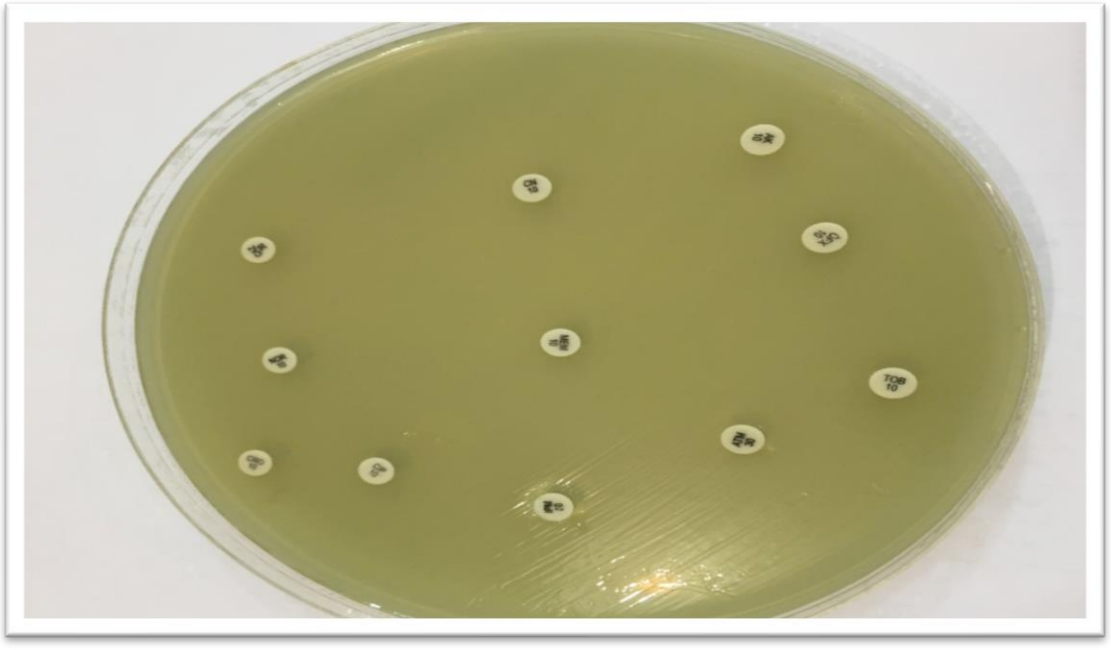
By present definition of MDR, 25(30.8%) of isolates were confirmed as MDR (Table 3-4), 6/25 (24%) of the them were resistant to three classes of antibiotics, and the remaining 19/25(76%) MDR isolates were resistant to four tested classes of antimicrobials. The most common agents involved in MDR were β-lactam/β-lactamase inhibitor combination, cefotaxime and gentamicin. However, no MDR isolates was resistant to imipenem.

Table (3-4):Multidrug resistance (MDR), Multidrug sensitive (MDS), extensive drug resistance (XDR) and pandrug resistance (PDR) of *Pseudomonas aeruginosa* isolates(n=81).

Type of resistance	No. of *P. aeruginosa* &(%)		No. of resisted Antibiotics classes(n=7)
MDR	25(30.8%)	6/25	3 classes
		19/25	4 classes
XDR	27(33.33%)	17/27	5 classes
		10/27	6 classes
PDR	9(11.11%)		7 classes
MDS	20(24.69%)		

Among these isolates, 27(33.33%) were resistant to 5 or 6 classes of antibiotics meeting criteria for XDR organisms. The majority of XDR isolates (17 of 27) were resistant to 5 classes of antibiotics, accounting for 63% of all isolates, (and 10 of 27) XDR isolates (37%) were resistant to 6 classes of antibiotics agents, while this 10(37%) of the isolates showed a pattern of XDR including meropenem, β-lactam/ β-lactamase inhibitor combination agents, cephalosporins and monobactams, isolates were susceptible only to imipenem.

Moreover, 9(11.11%) of isolates were resistant to all antibiotic classes (seven), and hence considered as PDR isolates. Figure (3-2) shows the isolate as PDR *P. aeruginosa*.



(Figure 3-2): Disk diffusion test for *P.aeruginosa* isolate apan-drug resistant (PDR).

In this investigation, antibiotic susceptibility testing of the *P. aeruginosa* isolates showed that 20(24.69%),25(30.8%), 27(33.33%), and 9(11.11%) of the isolates were MDS, MDR, XDR and PDR, respectively. Al-Wasity , (2018) reported that 14.5% of *P. aeruginosa* isolates from clinical samples obtained from hospitals in Baghdad/Iraq were pan drug resistant (PDR) and 29% of isolates were Multidrug sensitive (MDS) which is in a good agreement with our results that among 81 isolates 11.11% and 24.69% were pan drug resistant (PDR) and Multidrug sensitive (MDS) respectively .

Recent study reported significantly higher rate of extensive drug resistance -XDR *P. aeruginosa* in Diyala hospitals. By current definition of XDR, 33.33% of isolates were documented as XDR. Bacteria that are classified as XDR are epidemiologically significant due not only to their resistance to multiple antimicrobial agents, but also to their ominous likelihood of being resistant to all, or almost all, approved antimicrobial agents. In the medical literature XDR has been used as an acronym for several different terms such as extreme drug resistance , extensive drug resistance , and extremely drug resistant (Park *et al.*, 2009; Palavutitotai *et al.*, 2018).

There are currently very few reports on the clinical outcome of patients suffering from infection caused by PDR *P. aeruginosa*. These suggest that the mortality is high. In conclusion, the high incidence of MDR, XDR and PDR observed among *P. aeruginosa* isolates underlines the strict consideration in antibiotics use at Diyala hospitals. Therefore, it is important to perform antibiotic surveillance programs for appropriate empirical therapy and infection control practices.

3.6 Carbapenem-Resistant *Pseudomonas aeruginosa* Isolates

All 81 *P. aeruginosa* isolates were tested for carbapenems susceptibility using an initial screening test according to the guidelines of the Clinical and Laboratory Standards Institute (Patel *et al*., 2017). The isolates were initially screened for carbapenems susceptibility by disk diffusion method using imipenem, and meropenem (10 µg each) antibiotic disks. They were designated for imipenem and meropenem as susceptible if the inhibition zone diameter was 19 mm, intermediate if the inhibition zone diameter was 16-18 mm, and resistant if the inhibition zone diameter was 15 mm, as recommended by CLSI (2017).

Based on the results from susceptibility testing, 19 (23.45%) of *P. aeruginosa* isolates were found to be resistant to at least one of carbapenems Figure (3-3). Susceptibilities of the isolates to imipenem and meropenem are listed in table (3-3). Imipenem showed better activity (80.24%) than meropenem (61.72%) in study period.

Resistance for carbapenems by disk diffusion was originating in 9(11.11%) isolates for both meropenem and imipenem, and in 10(12.34%) isolates for meropenem alone, respectively. However, cross-resistance between meropenem and imipenem was found in 9 of 19 isolates resistant to both meropenem and imipenem. The remaining 10 isolates of *P. aeruginosa*, formally resistant against meropenem were found susceptible to imipenem.

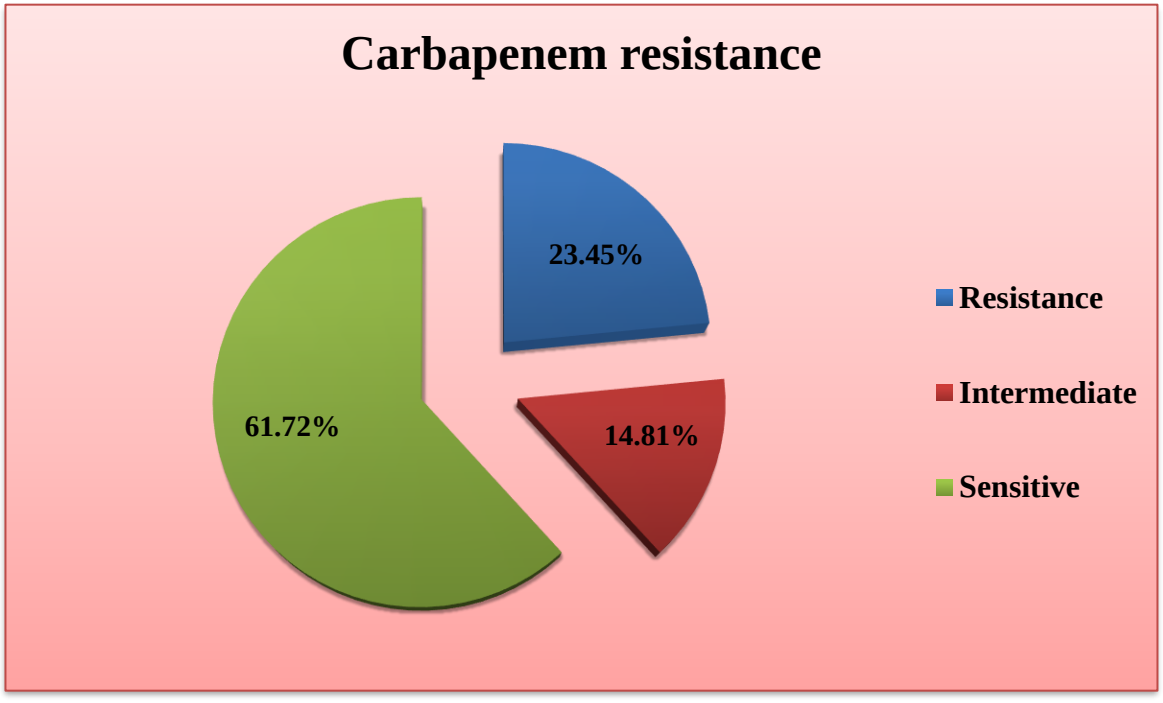


Figure (3-3): Carbapenem (Imepenem and Meropenem) susceptibility rates of *P. aeruginosa*

There is a limited literature available regarding the prevalence of resistance to carbapenems in *P. aeruginosa* from clinical isolates in Diyala province. Azeez and Bakr ,(2019) reported that 28% of *P. aeruginosa* isolates from clinical samples obtained from hospitals in Erbil/Iraq were resistant to carbapenem which is in a good agreement with our results that among 81 isolates 23.45% were resistant to Imipenem and Meropenem. While previous data in Karbala Hospitals suggest that 68.34% of *P.aeruginosa* clinical isolates were resistant to carbapenems (Shilba *et al.*, 2015). In previous reports published from Wasit/Iraq by Hussein *et al.* (2018) reports of the resistance of *P. aeruginosa* to carbapenems was 34.95% .

Accordingly, this finding on carbapenem resistance among the *P. aeruginosa* is an emerging phenomenon of great clinical and public health importance. Similar studies should be suggest in each part of Iraq to avail the prevalence and enhanced measures to control the spread of this resistant in these isolates. However, imipenem and meropenem were found to be the most effective antibiotics among all the antibiotics used inthis

study. On the other hand, overuse of carbapenems in Diyala hospitals may be contribution to growing phenomenon of MDR.

Carbapenem resistance towards the gram negative microorganisms especially *Pseudomonas aeruginosa* is alarming and on-going public health problem all over the world. It may be intrinsic or acquired through transcription of genes among microorganisms. These genes are spreading rapidly and are responsible for serious out-breaks. So selection of antibiotics is limited to treat these resistant cases (Bakhat *et al*.,2019).

Carbapenems have been extremely prescribed for treatment of *P. aeruginosa* infections. Carbapenemase production has emerged as the main mechanism of carbapenem resistance among clinical isolates of *P. aeruginosa*. However, loss of *Opr*D has also contributed for carbapenem resistance, especially conferring resistance to imipenem. Acquisition of mutations,insertions, and/or deletions in the *oprD* gene are the most common mechanisms of *Opr*D inactivation as well as down-regulation of *oprD* transcription(Campana *et al*.,2017).

3.7 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Imipenem,Ceftazidime and Gentamicin against Resistant *P. aeruginosa*

Imipenem,Ceftazidime and Gentamicin were selected as the antibiotics to detect the MIC and MBC against 19 carbapenem resistant *P. aeruginosa* depended on the break point from CLSI (2017).

Determination of MIC and MBC was done by using Muller Hinton broth in tow fold dilution method. MIC values for each isolate were determined by choosing the lowest concentrations in which no growth occurs. Additionally, the minimal bactericidal concentration (MBC) values were determined by sub -culturing the content of each tube without any growth, on the Mueller Hinton agar medium and looking for any bacterial

growth. An isolate was characterized as resistant if the MIC was greater than the breakpoint as defined by CLSI (2017), while it will be susceptible if it is less than the breakpoint. All the 19 isolates of the current study showed high degrees of resistance.

Table (3.5) shows that the MICs of ceftazidime were ranged between (64 - 512 µg /ml) and MBC was (128- >1024) . This result agreed with the result of Pourakbari *et al*., (2016) which reported that the rate of resistance to ceftazidime was high that showed high concentration in MIC and MBC values.

For gentamicin, the break point for this antibiotic was ≥16 µg/ml and the lowest MIC was 64µg/ml for the isolate Pa12, but in some cases the MIC elevated to reach 1024 µg/ml as with three isolates (Pa5-Pa8 and Pa19), while MBC was ranged between (256 - >1024 µg /ml) . In the research done by Bubonja-Sonje *et al,*.(2015) they illustrated that the values of MIC for this antibiotic were between 1.5->256 µg/ml and rate of resistance reached 34% and these result are much lower than the percentage of this study.

MIC of imipenem showed a varied range from 2 µg /ml for 256, with a maximum of 256 µg /ml for one isolate (Pa10) and MBC was (4- 512 µg /ml). Rostami *et al*., 2018 found that the rate of resistant to imipenem 78.5% and the values of MIC for this antibiotic were between 8-≥256 µg/ml.

The reason of high value of MIC and MBC the highly production of beta lactamase enzyme that breakdown the ring of B-lactam and presence of resistance genes that play a role in inhibition the activity of antibiotic (Bush and Jacoby 2010; Gonçalves *et al*., 2017).

Table (3-5): The MIC & MBC values of Imipenem , Ceftazidime and Gentamicin against 19 carbapenem resistant *P. aeruginosa* .

Antibiotic / No. of the Isolates	Ceftazidime		Gentamicin		Imipenem	
Breakpoints µg/ml	**≥32**		**≥16**		**≥8**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
ATCC27853 *Pseudomonas aeruginosa*	4	8	2	8	0.5	4
Pa1	512	>1024	256	1024	4	16
Pa2	256	1024	512	>1024	128	512
Pa3	256	512	512	1024	16	64
Pa4	64	128	256	512	4	16
Pa5	512	>1024	1024	>1024	32	64
Pa6	256	512	256	1024	8	16
Pa7	512	1024	512	>1024	128	512
Pa8	512	512	1024	>1024	128	256
Pa9	128	265	256	512	4	16
Pa10	256	512	512	>1024	256	512
Pa11	64	256	128	512	4	16
Pa12	512	1024	64	256	2	4
Pa13	128	256	256	1024	16	32
Pa14	64	128	512	>1024	8	16
Pa15	256	1024	128	256	2	8
Pa16	512	>1024	512	>1024	32	128
Pa17	64	128	128	512	4	16
Pa18	128	512	128	512	2	8
Pa19	256	512	1024	>1024	16	32

3.8 Antibacterial Activity of Zinc Oxide Nanoparticles

3.8.1Determination the Minimum Inhibitory Concentration (MIC) of ZnO Nanoparticles against *P. aeruginosa*

Overuse or misuse of antimicrobials has led to the development of multi-drug resistant bacteria. To overcome the limitations of conventional synthetic antimicrobial compounds, nanotechnology represents an alternative strategy in developing alternative antimicrobial agents that can efficiently kill bacterial cells and display immense potential for use in both medical and veterinary applications.

The inhibitory effects of different concentrations of ZnO NPs on *P. aeruginosa* isolates were examined and described in table (3.6). MIC of ZnO NPs is ranged between (325 -5200 µg/ml) on *P. aeruginosa* isolates. This result was agreed with the result of Masoumi and Shakibaie (2018) reported that the values of MIC for ZnO NPs were between (80- ≥2600 µg/ml) that showed high concentration in MIC and MBC values against *P. aeruginosa*.

In the research done by Alanbare *et al.,* (2014) the results showed that the concentrations (500,1000, 5000, 10000, 20000) µg/ml were lethal to *pseudomonas aeruginosa*, while (100 and 75) µg/ml were inhibitors and the concentrations (50,25) µg/ml were not effective. While Bohan *et al.,* (2018) showed that the results of Zinc Oxide Nano Particles against *pseudomonas aeruginosa* explain ability of its to inhibition growth of bacteria in different rate of bacteriostatic when used in different concentration (800-2400 µg/ml).

The antibacterial mechanisms of ZnO NPs include binding to and damaging the bacterial membrane, penetrating into the bacterial, and generating reactive oxygen species (ROS). Therefore, it is probable that ZnO NPs by increasing the permeability of cell membrane may cause the enhancement of antibiotic efficacy. Another explanation for these findings would be that ZnO NPs may interfere with pumping activity of efflux

systems. The generation of hydrogen peroxide is a main factor of the antibacterial activity (Bayroodi and Jalal ,2016).

Table (3.6): The MIC values of ZnO NPs against 19 carbapenem resistant *P. aeruginosa*.

No. of Isolates	MIC of ZnO NPs	No. of Isolates	MIC of ZnO NPs
Pa1	1300	**Pa11**	2600
Pa2	650	**Pa12**	325
Pa3	1300	**Pa13**	1300
Pa4	2600	**Pa14**	2600
Pa5	650	**Pa15**	2600
Pa6	2600	**Pa16**	5200
Pa7	650	**Pa17**	1300
Pa8	1300	**Pa18**	1300
Pa9	5200	**Pa19**	650
Pa10	1300	**ATCC27853 *Pseudomonas aeruginosa***	40.6

3.9 Phenotypic Determination of β-Lactam-Resistance Mechanism

3.9.1 Detection of extended spectrum β-Lactamase production

The carbapenem-resistant *P. aeruginosa* isolates (19) were tested for ability to produce ESBL enzymes The isolates were screened for ESBL production by disk diffusion method using ceftazidime, cefotaxime, ceftriaxone and azetreonam (30 µg each) antibiotic disks. According to the CLSI (2017), the isolate is considered to be potential ESBL producers, if the inhibition zone of cefotaxime and ceftazidime was 14

mm, ceftriaxone was 13 mm, and aztreonam was 15 mm. Frequency of ESBLs producing isolates by disk diffusion assay are summarized in table (3.7). Rrsistance of cefotaxime, ceftriaxone, Ceftazidime and azetreonam effect were observed in all 19 (100%) isolates tested. Primary phenotypic test revealed that ESBL phenotype was recognized in all carbapenem-resistant *P. aeruginosa* isolates (100%) which expressed resistance to any of the expanded-spectrum cephalosporins and monobactam. High rate of ESBLs-producing *P. aeruginosa* isolates were recovered from clinical samples in Baquba/Diyala.

ESBL production was also screened for using the double disk synergy test (DDST) as a standard disk-diffusion assay on Mueller-Hinton agar. Results revealed that the isolates were unable to produce ESBL as the increased zone size towards the ticarcillin-clavulanic acid disk (Table 3.7). This may be due to the simultaneous production of other type of β -lactamase that could mask presence of the ESBL **(**AmpC β-lactamases or inhibitor resistant cephalosporinases). However, the study indicated that no correlation was found between the results obtained disk diffusion and DDST method. This result agreed with the result of Al-Shara, 2013 and Abbas *et al*., 2018 reported that the phenotypic analysis showed that none of their isolates was an ESBL producer.

Table (3-7): Comparsion between initial screening and double disk synergy test for ESBL production by 19 carbapenem-resistant *P. aeruginosa* isolates

No. (%) of Resistance by disk diffusion test				**Double disk synergy test positive NO. (%)**
Ceftriaxone	Ceftazidime	Cefotaxime	Aztreonam	
19(100%)	19(100%)	19(100%)	19(100%)	0(0.0%)

3.9.2 Detection of Metallo β-Lactamase MBLs production

All *P. aeruginosa* isolates that were resistant to carbapenems (n=19) were further investigated for the presence of chromosomal or plasmid-mediated MβLs genes. Combined Imipenem-EDTA disc method performed for detection of the ability of *P. aeruginosa* isolates to produce MBL. That is responsible for their resistance to beta-lactam antimicrobial agents like Imipenem and Meropenem. Among 19 isolates of *P. aeruginosa* 16 (84.21%) of the isolates were MBL producers, the inhibition zone with Imipenem-EDTA is more than 7 mm than the IMP disc alone. The remaining *P. aeruginosa* isolates 3 (15.78%) were non-MBL producers Figure (3-4).

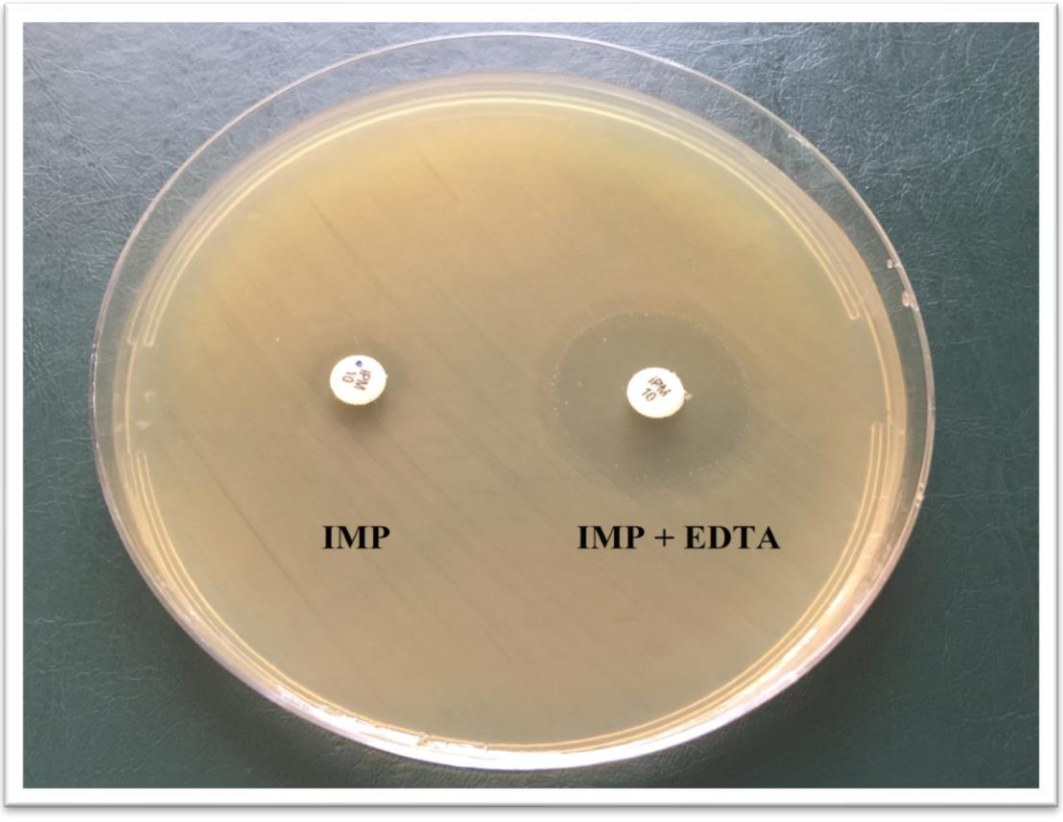


Figure (3-4): Combined disc diffusion test MBL positive

In the present study, 84.21% of carbapenem resistant *P. aeruginosa* isolates have Metallo β-lactamase, and this result agreed with Al-Shara, (2013) who found that 78% of carbapenem resistant *P. aeruginosa* isolates from patients have MBL enzyme, also

agreed with (Kazeminezhad *et al*., 2017) who demonstrate 73% of carbapenem resistant isolates in have MBL enzyme inTehran/Iran. Early detection of these MBL producing isolate in a routine laboratory could help to avoid treatment failure, as often the isolates producing this enzyme show a susceptible phenotype in routine susceptibility testing.

3.10 Molecular Study of *Pseudomonas aeruginosa* DNA

The genetically study is boosting for the conventional detection and investigation methods, with Polymerase Chain Reaction(PCR) which plays an important role as a powerful tool in clinical microbiology studies and has been widely applied to detect bacteria and genes of interest(Thong *et al*., 2011) .

3.10.1 Total DNA Extraction of *Pseudomonas aeruginosa* Isolates

Using a genomic DNA purification kit (Promega,USA), for total genomic DNA extracted from *P. aeruginosa* isolates. Extraction genomic DNA from 19 carbapenem-resistant isolates that was confirmed qualitatively as bands by gel electrophoresis.

Determination of DNA concentration and purity were done using the Quantus Fluorometer and Nano drop spectrophotometer device previously mentioned. The results indicated that DNA concentrations of the extracts were variable ranging from 59 ng/ µl to 183ng/ µl. It was also observed that the purity of DNA extracts was satisfactory and ranging from 1.73 to 1.97 according to the value rate of 280/260nm. The output is dependent on culturing methods, bacterial category, amount of pellet and type of extraction kit, all these have an affect on quality and properties of nucleic acid. That is, most of molecular practical methods indicated to more easy and adequate of DNA extraction from gram negative than positive and this applies to recent ready extraction kits(Knudsen *et al*., 2016).

3.10.2 Detection 16S rRNA gene by conventional PCR

Genotypic identification results revealed that all 19 isolates (100%) were *P.aeruginosa* which showed expected amplicons size 956bp for housekeeping gene 16S rRNA. Figure(3.5) illustrated shine bands of positive isolates compared with 100bp. DNA ladder.The genotypic analysis via PCR reaction with specific 16S rRNA, which ended confusion in the diagnosis confirmation process, although the VITEK 2 system proved that 19 specimens were within *P. aeruginosa*, and the accurate conclusive outcome gotten from PCR-technique detection through 16S rRNA gene . There were 19 isolates belong to species of *P. aeruginosa*, so, there is no difference between VITEK 2 system test and molecular diagnosis or simple and not confusing.

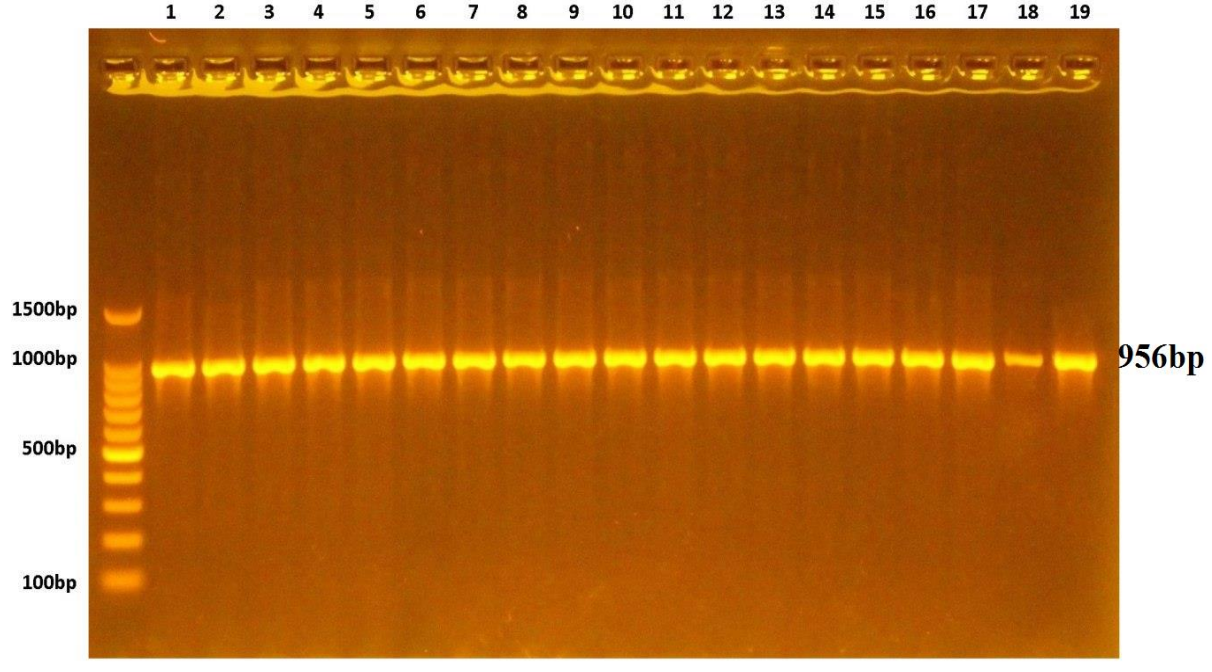


Figure (3-5): Gel electrophoresis of amplified PCR product for the detection of 16SrRNA gene (956bp) run on 1%agarose (90 min at 70 volt), stained with ethidium bromide, lane 1-19 *P.aeruginosa* isolates; M:Marker DNA ladder(100bp).

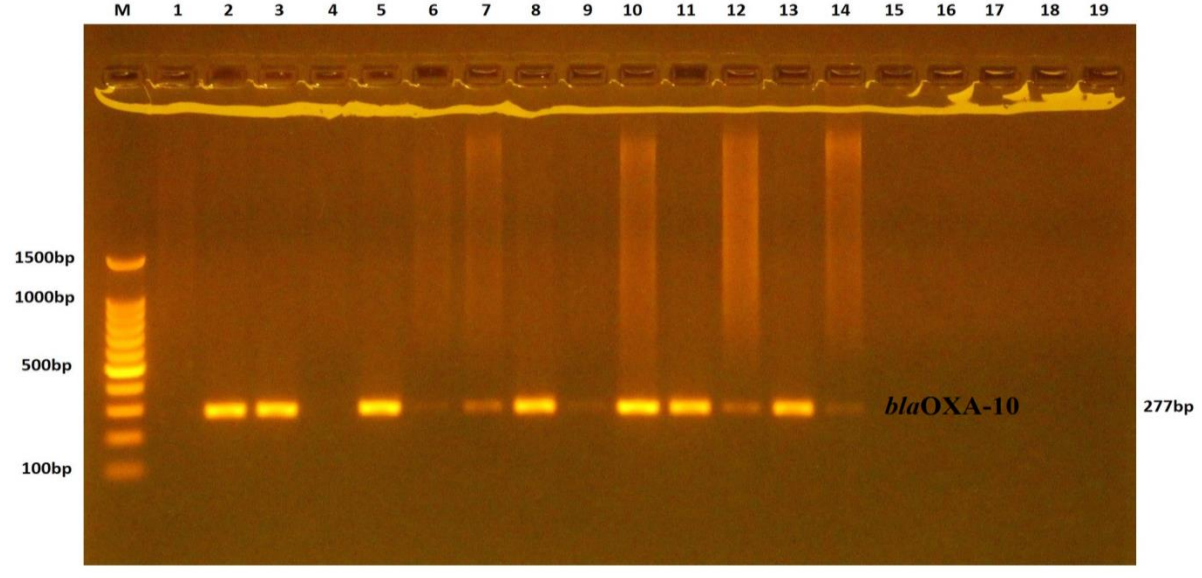
A 16S ribosomal DNA sequence has long been used as a taxonomic " gold standard " in determining the phylogenies of bacterial species(Spilker *et al*., 2004), Polymerase Chain Reaction is a better alternative than conventional methods, as it targets the DNA

of an organism. The conventional methods used for diagnosis are time consuming, costly, and labor-intensive(Teh *et al*., 2008).

3.10.3 Molecular Characterization of ESβL genes *bla*$_{PER}$ and *bla*$_{OXA-10}$

This study was carried out in order to detect ESBL genes in 19 carbapenem-resistant *P. aeruginosa* isolates. ESBLs genes OXA-10 and PER was screened by PCR technique for isolates. The results of gel electrophoresis for PCR product by using specific primers for this genes showed that 12(63.15%) isolates were positive for *bla*$_{OXA-10}$ gene as shown in Figure (3-6). Results revealed that gene *bla*$_{OXA-10}$ was the most spread gene. Noteworthy, none of the 19 carbapenem-resistant isolates of *P. aeruginosa* had *bla*$_{PER}$ ESBL gene.

The percentage of *bla*$_{OXA-10}$ gene in the current study was higher than previous study in some Hospitals in Bagdad Governorate it was noted that *bla*$_{OXA-10}$ gene appeared in 19.4% among *P.aeruginosa* isolates (AL-Rubaye *et al*., 2015).While a study in Southwest of Iran by Alam *et al*,(2018)noticed that among (21) isolates of *P.aeruginosa* collected from hospitalized patients, 19 (90.5%) were positive for *bla*$_{OXA-10}$.



Figure(3-6): Gel electrophoresis of amplified PCR product for the detection of ESβLs *bla*$_{OXA-10}$ gene (277bp) run on 1%agarose (90 min at 70 volt), stained with ethidium bromide, lane 1-19 *P.aeruginosa* isolates ; M:Marker DNA ladder (100bp); Lanes 2,3,5,6,7,8,9,10,11,12,13,14 positive for *bla*$_{OXA-10}$.

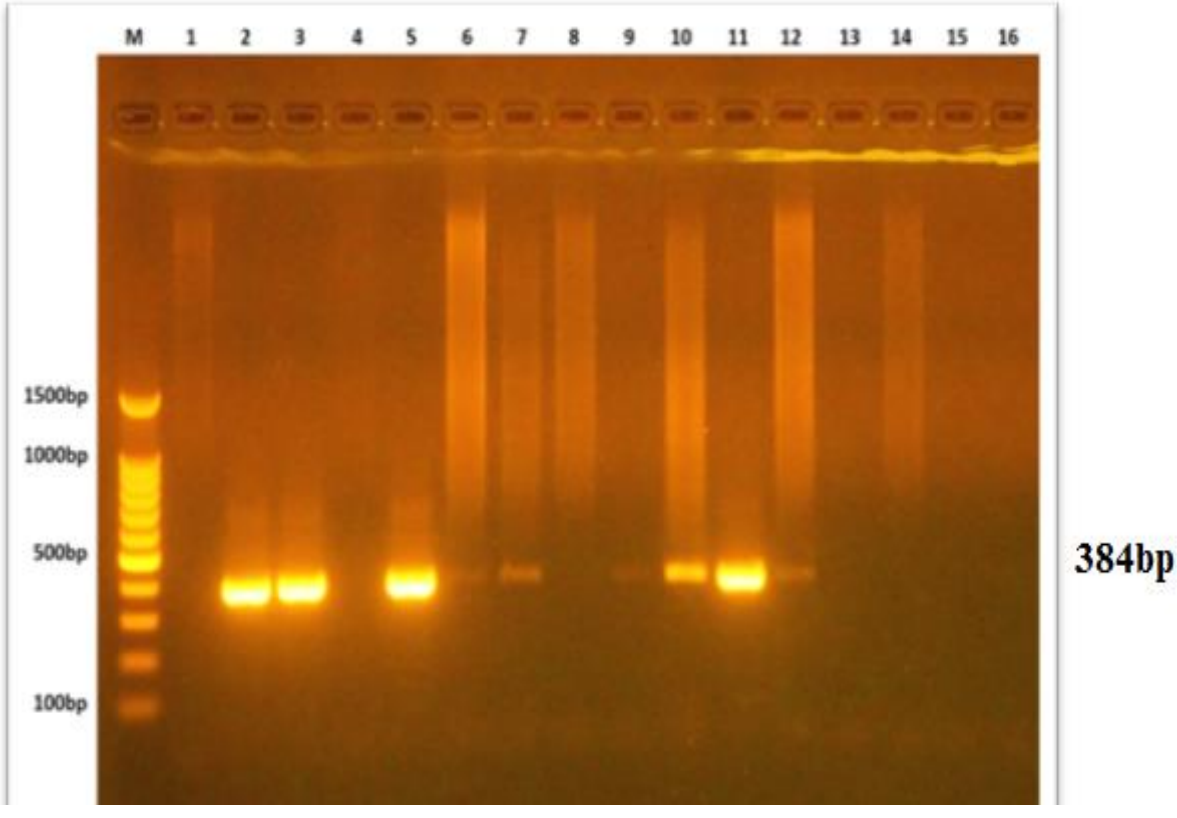
The present study underlines the unique problem that the presence of ESBL has led to widespread consumption of imipenem, but that the emergence of MBLs and their broad spectrums and unrivalled drug resistance is creating a therapeutic challenge for clinicians and microbiologists. Hence, the present study suggests that the detection of ESBL and MBL in *P. aeruginosa* should be a routine practice. Moreover, it recommends a routine surveillance on antibiotic resistance in the hospital.

3.10.4 Molecular Characterization of MBL genes *bla*$_{VIM}$, *bla*$_{IMP}$ and *bla*$_{NDM}$

Among 16 phenotypic Metallo β-lactamase isolates the results achieved by using PCR revealed that 9 (56.25%) isolates have *bla*$_{VIM}$ genes as in Figure (3.7), while 4 (25%) isolates carried *bla*$_{NDM}$ genes Figure (3-8). No *bla*$_{IMP}$ was detected among Imipenem resistant strains in this study. The current results showed that 56.25% percentage of carbapenems resistant *P. aeruginosa* isolates have *bla*$_{VIM}$ gene, the percentage of *bla*$_{VIM}$ gene in the current study was higher than previous study in Saudi Arabia in which *bla*$_{VIM}$ appeared in 29.4% of isolates (Al-Agamy *et al*., 2016). In another study in Some Hospitals in Bagdad Governorate (60%) of isolates carried *bla*$_{VIM}$ genes, this result agreed with the current study (AL-Thwani ,2013). While study in Wasit province by Hussein & Shamkhi ,(2018) showed that 94.44% percentage of carbapenems resistant *P. aeruginosa* isolates have *bla*$_{VIM}$ gene .This result disagreed with the current study.

The percentage of *bla*$_{NDM}$ gene in the current study was lower than previous study in Wasit province by Hussein *et al*.,(2018) which showed that 50% percentage of carbapenems resistant *P. aeruginosa* isolates have *bla*$_{NDM}$ gene. While a study in Najaf (Al-Shara, 2013) showed that only 2 (5.6%) isolates harbored *bla*$_{NDM}$ gene (23). In Slovakia, a study was reported *bla*$_{NDM}$ gene in 6 isolates(20%). This result agreed with the current study(Kulkova *et al*., 2015).

In our study, 62.5% of MBL isolates carried MBL genes this is agreed with Esmaeili *et al*., (2019) who found that 68.6% of isolates carried MBL genes. The *bla* genes are located in class1 integrons as a gene cassettes and have been identified on plasmid with different replicon types ,increasing the possibility of dissemination and linkage to other antibiotic resistance genes. However, this constitutes the first report on prevalence and detection of *bla*$_{VIM}$ and *bla*$_{NDM}$ genes in *Pseudomonas aeruginosa* in Diyala province.



Figure(3-7): Gel electrophoresis of amplified PCR product for the detection of MβL *bla*$_{VIM}$ gene (384bp) run on 1%agarose (90 min at 70 volt), stained with ethidium bromide, lane 1-16 *P.aeruginosa* isolates;M:Marker DNA ladder(100bp); Lanes2,3,5,6,7,9,10,11,12 positive for *bla*$_{VIM}$.

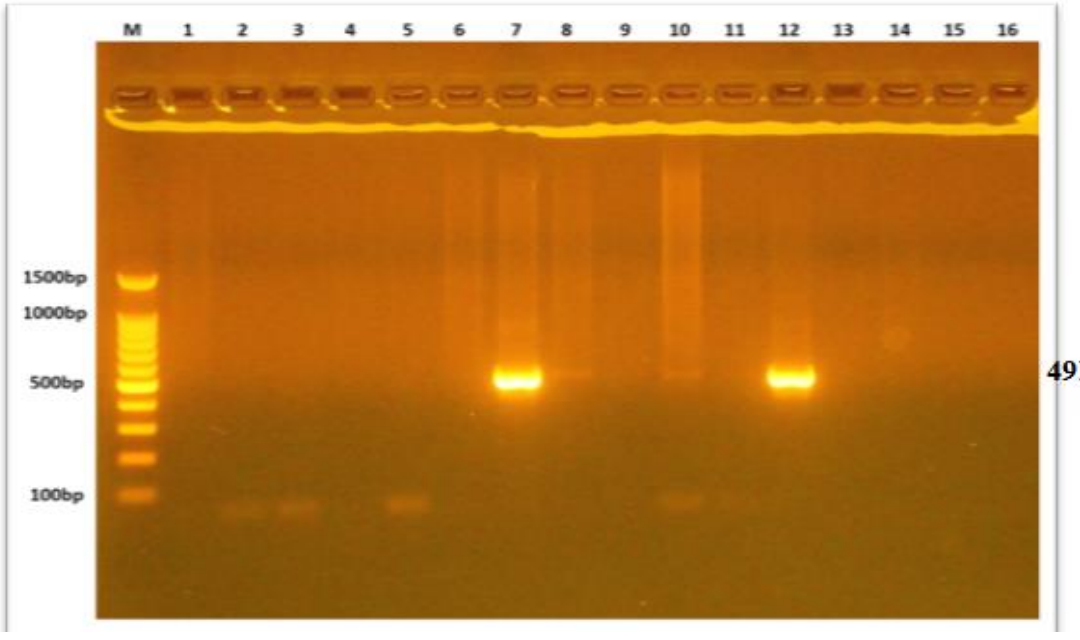


Figure (3-8): Agarose Gel electrophoresis of amplified PCR product for the detection of MβLs *bla*$_{NDM}$ gene (491bp) run on 1%agarose (90 min at 70 volt) , stained with ethidium bromide, lane 1-16 *P. aeruginosa* isolates;M: Marker DNA ladder (100bp); Lanes 7,8,10, 12 positive for *bla*$_{NDM}$.

3.10.5 Molecular Detection of Aminoglycoside-Modifying Enzyme (AME) Genes *aac(6')-Ib, aac(3')-II and ant(4')-IIb*.

In the current study, the result of AME genes[*aac(6')-Ib, aac(3')-II and ant(4')-IIb*] Aminoglycoside-Modifying Enzyme(AME) genes were detected in carbapenems resistant *P.aeruginosa* (19) selected isolates using specific primers for Aminoglycoside O-nucleotidyltransferases *ant(4')-IIb* (462bp), and two Aminoglycoside N-acetyltransferases *aac(6')-Ib* (304bp) and *aac(3')-II* (212bp) genes according to Dakhl and Alwan,(2015) .

The genes coding for the modifying enzymes are either present alone or in combination between two genes or three genes [but none of the isolates in the current study can be seen and observed to contain the three detected genes]. Table (3.8) shows the isolates harboring these genes and their percentage.

Table (3-8) Frequency of AMEs Genotypes Among *Pseudomonas aeruginosa* Isolates

Genes	No. of Isolates out of 19 & %
Whithout any AMEs genes	1(5.26%)
aac(6')-Ib	18 (94.73%)
aac(3')-II	1 (5.26%)
ant(4')-IIb	2 (10.52%)
aac(6')-Ib, aac(3')-II	1(5.26%)
aac(6')-Ib , ant(4')-IIb	2 (10.52%)
aac(6')-Ib, aac(3')-II and *ant(4')-IIb*	0 (0%)

Results by Uniplex PCR technique showed that the *aac(6')-Ib* was the most prevalent AME genes since it was found in 18/19 (94.73%) of the isolates figure (3-9), followed by *ant(4')-IIb* and *aac(3')-II* when the rates were 10.52% (2/19), 5.26%(1/19) respectively . Figures (3.10) and (3.11) illustrate PCR products for *aac(3')-II* and *ant(4')-IIb* genes.

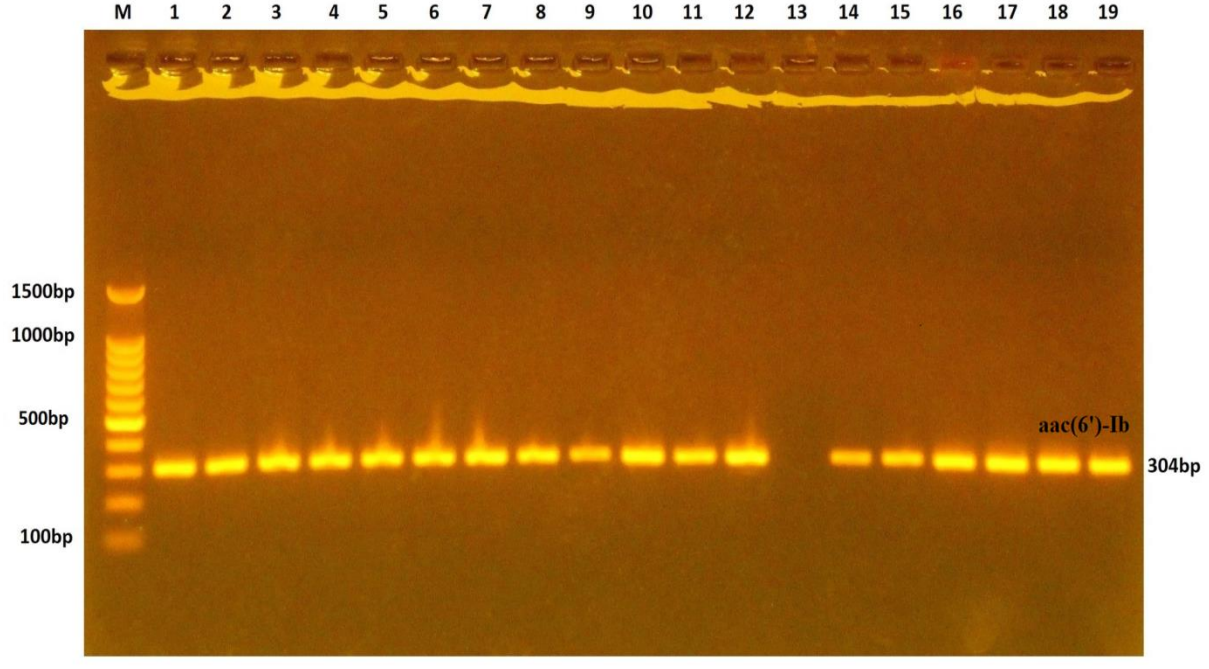


Figure (3-9): Agarose Gel electrophoresis of amplified PCR product for the detection of aac(6')-Ib gene (304bp) run on 1%agarose (90 min at 70 volt) , stained with ethidium bromide, lane 1-19 *P. aeruginosa* isolates;M: Marker DNA ladder (100bp); All Lanes positive for *aac(6')-Ib* gene except the lane 13 was negative result.

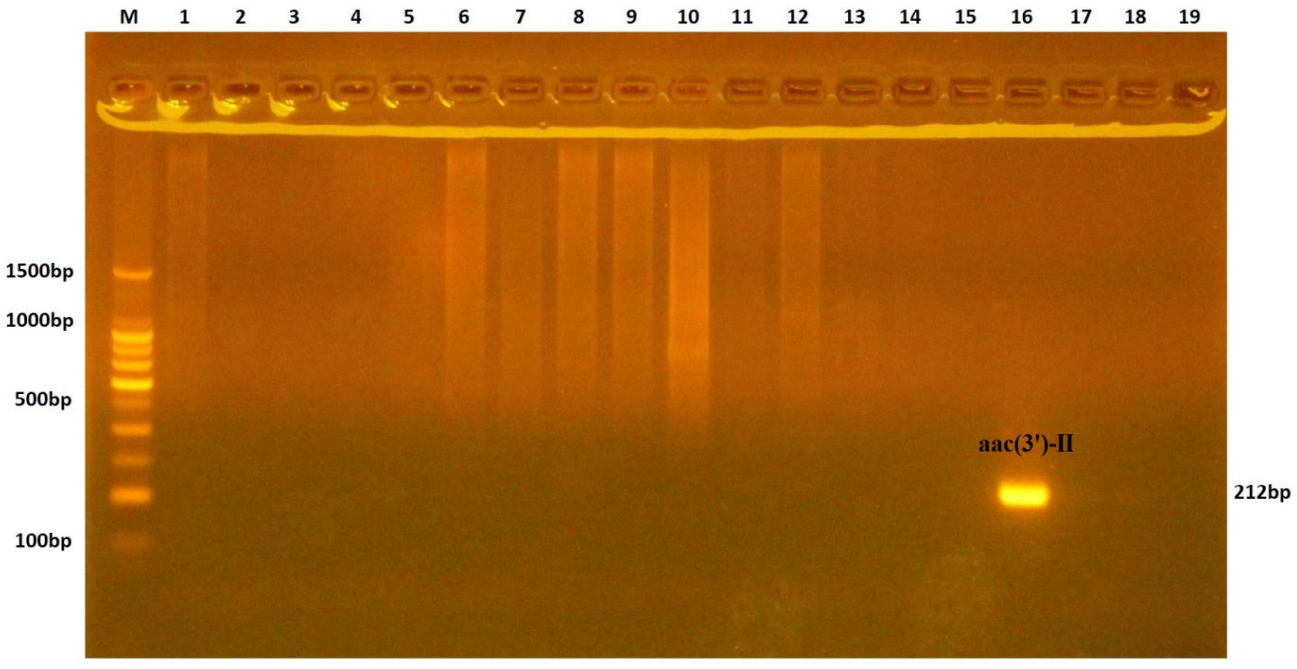


Figure (3-10): Agarose Gel electrophoresis of amplified PCR product for the detection of *aac(3')-II* gene (212bp) run on 1%agarose (90 min at 70 volt) , stained with ethidium bromide, lane 1-19 *P. aeruginosa* isolates;M: Marker DNA ladder (100bp); only Lane 16 positive for *aac(3')-II* gene.

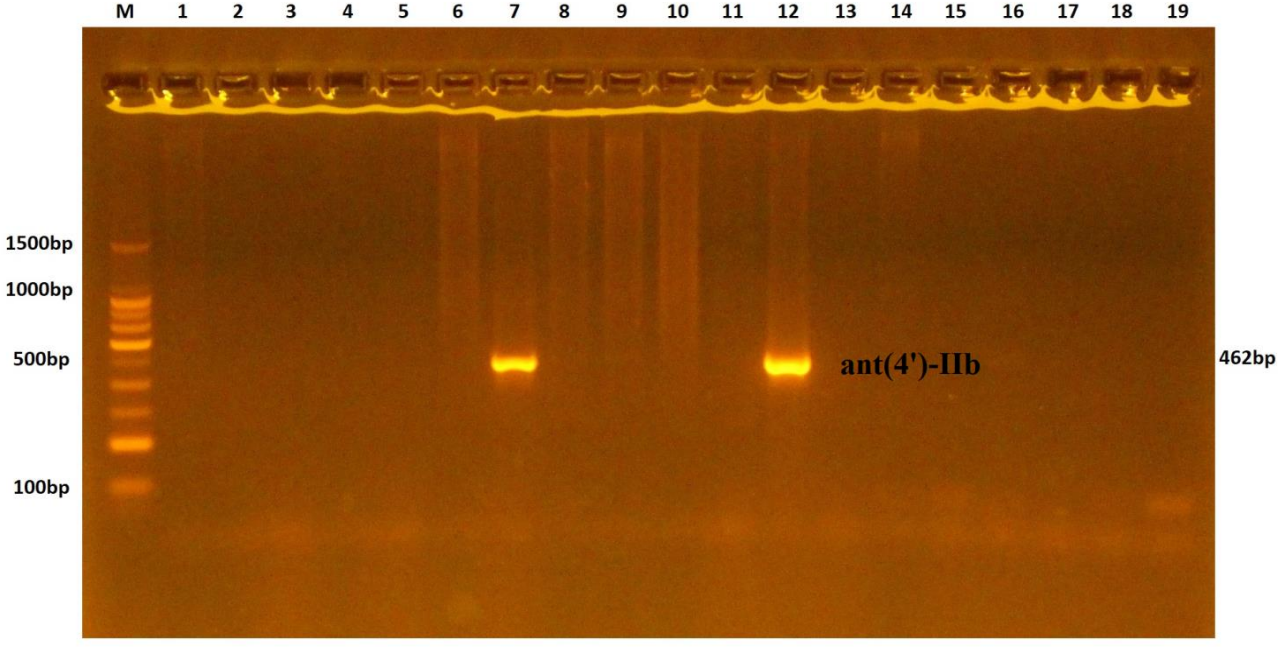


Figure (3-11): Agarose Gel electrophoresis of amplified PCR product for the detection of ant(4')-IIb gene (462bp) run on 1%agarose (90 min at 70 volt) , stained with ethidium bromide, lane 1-19 *P. aeruginosa* isolates;M: Marker DNA ladder (100bp); only Lane 7 and 12 positive for *ant(4')-IIb* gene.

The percentage of *aac(6')-Ib* gene in the current study is very high as compared with the results of Al-Jubori *et al.* (2015) in Baghdad\ Iraq and El-Badawy *et al.*(2019) in Saudi Arabia who illustrated that *aac(6')-Ib* gene were detected in 42.8% and 71.8 % of *P.aeruginosa* isolates respectively.

For the second modifying genes *ant(4')-IIb* , its percentage in the current study was lower than previous study in AL-Diwanyia province, Iraq by Dakhl and Alwan, (2015) which showed that 21.8% percentage of *P. aeruginosa* isolates have *ant(4')-IIb* gene. While Song *et al*. (2018) found the prevalence rate of *ant(4')-IIb* gene reached 71.4% in *P.aeruginosa* .This result is relatively high as compared with the current ratio. While Ndegwa *et al*. (2012) in Kenya illustrated that none of their isolates were harbouring *ant(4)- IIb* gene. The percentage of gene *aac(3')-II* in *pseudomonas areugenosa* is 5.26% in current study. In China, a study was reported by Song *et al*,(2018) found *aac(3')-II* gene in 19 isolates(38.8%), this result disagreed with current study .

The differences in the distribution of modifying enzymes may be derived from differences in aminoglycoside prescription patterns,and the selection of bacterial population or geographical differences in the occurrence of aminoglycoside resistance genes .

Several of the *P. aeruginosa* isolates showing phenotypic resistance to aminoglycosides evaluated in this study did not test positive for the resistance genes evaluated. This suggests that other genes, not tested here, which confer resistance to aminoglycosides, may be present in these isolates. There are also other mechanisms that could lead to aminoglycoside resistance, such as: changes in the permeability of the external membrane, active efflux systems, and alterations of the 30S ribosomal subunit conferred by mutations(Teixeira *et al*., 2016).

3.10.6 Molecular Detection of Efflux Pumps Genes *MexY, MexB, MexD* and *MexF*.

In the current study, the result of efflux pumps genes (*MexY, MexB, MexD* and *MexF*) achieved by two conventional PCR techniques: uniplex and multiplex PCR, for 19 selected carbapenems resistant isolates showed that all isolates 19 /19 (100%) were positive for one or more efflux pumps.

Uniplex results by PCR technique showed that 19/19 (100%) of isolates were positive for *MexY* gene as in figure (3-13). Based on the results from Multiplex PCR, 18 isolates (94.7 %) have *MexB* and *MexF* genes, while 17 isolates (89.47%) have *MexD* gene figure (3-14).This may indicate the prevalence of these types of resistance in the current isolated bacteria .

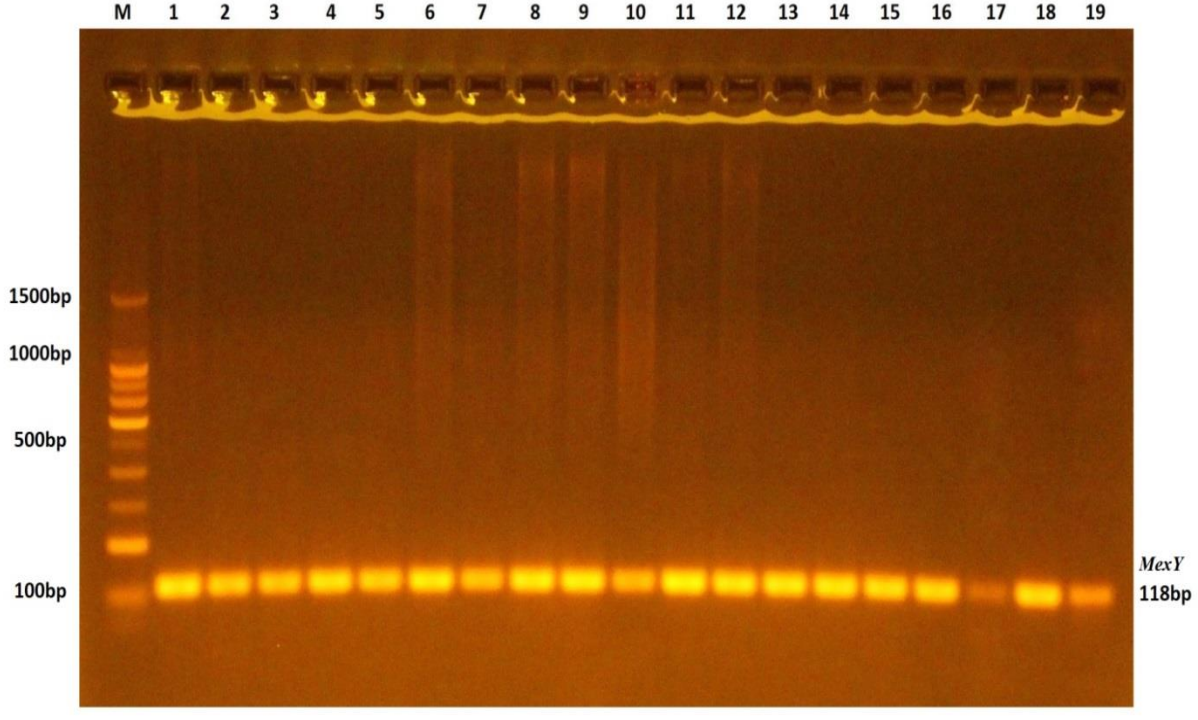


Figure (3-12): Agarose Gel electrophoresis of amplified PCR product for the detection of *MexY* gene (118bp) run on 1%agarose (90 min at 70 volt) , stained with ethidium bromide, lane 1-19 *P. aeruginosa* isolates;M: Marker DNA ladder (100bp); All Lanes positive for *MexY* gene.

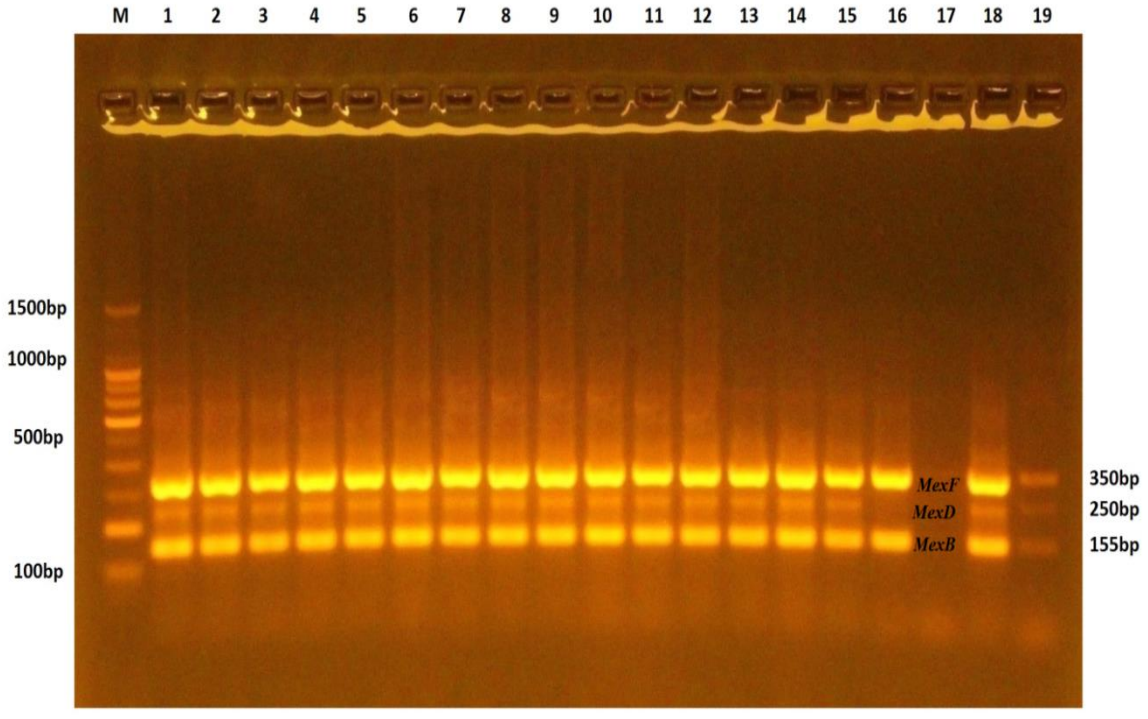


Figure (3-13): Agarose Gel electrophoresis of amplified multiplex PCR product for the detection of *MexF*(350bp), *MexD*(250bp) and *MexB*(155bp) genes run on 1%agarose (90 min at 70 volt) , stained with ethidium bromide, lane 1-19 *P. aeruginosa* isolates;M: Marker DNA ladder (100bp).

MexY gene was detected in 8/28 (28.5%) isolates in a study performed by Al-Jubori *et al*. (2015) and this result disagreed with the current study. Al-Marjani *et al*. (2015) investigated a survey of efflux pump gene expression of *P. aeruginosa* in 27 colistin resistant isolates isolated from Iraqi patients. Their investigation included PCR assay for determining the known genes expression of efflux pump as well as *mexY* gene, which illustrated that the prevalence rate of *mexY* was 88.9% and this result was approximated in line of the current study.

In the current study, it could be noticed that the correlation between higher MICs values and efflux genes expression was frequently found in combination with modifying enzymes genes and ESBLs genes. The results revealed that 94.7% of the isolates (18/19) were harboring both AMEs and efflux genes and it could be seen that most of isolates harboring more than one of the efflux pump genes (17,18 and 19 for *mexD,MexB,MexF*

and *mexY* genes respectively) in corporation with one or more of AMEs. The results revealed that (63.15%) of the isolates (12/19) were harboring both ESβLs and efflux genes and it could be seen that most of isolates harboring more than one of the efflux pump genes in corporation with *bla*OXA-10 of ESβLs.

Usually, aminoglycoside acetyltransferase (AAC) enzyme encoded by *aac(6')-Ib* and *aac(3')-II* genes have high activity against gentamicin specially the first gene .It can be noticed that all isolates harboring *aac(3')-II* gene can resist gentamicin except some of the isolates which still can resist gentamicin but devoid this gene. It is possible that the efflux system gave a good reason for this resistance because all these isolates were harboring *mexY* gene whether alone or in corporation with the *aac(6')-Ib* gene detected in 18 isolates and all these isolates were resistant to gentamicin .

The current results showed that 94.7 % percentage of resistant *P. aeruginosa* isolates have *MexB* gene, the percentage of *MexB* gene in the current study was higher than previous study in Iran which noted that *MexB* appeared in 53.3 % of isolates (Pourakbari *et al*., 2016). In another study in Some Hospitals in Baghdad Governorate (100%) of isolates carried *MexB* gene. This result agreed with the current study (Abd ,2018).

At present, the efflux pump has been recognized as one of the significant complexes involved in resistance to most of the classes of antibiotics (Shigemura *et al*.,2015). There are rare reports on prevalence of efflux pump genes in our Provence. In the present study the increased expression level of *MexD-OprJ* genes of efflux pump simultaneously was 89.47% which was relatively more than Murugan *et al*. (2017) findings in India which noted that *MexD* gene appeared in 43% of isolates.

In this study, 18 of 19 patients (94.7 %) showed an increased expression level of efflux pumps *MexF-OprN* genes that was higher than the previous study in Spain which reported that *MexF* appeared in 4.2% of isolates (Cabot *et al*., 2011). The multidrug efflux system MexEF-OprN is produced at low levels in wild-type strains of *Pseudomonas aeruginosa*. However, in so-called *nfxC* mutants, mutational alteration of

the gene *mexS* results in constitutive overexpression of the pump, along with increased resistance of the bacterium to chloramphenicol, fluoroquinolones, and trimethoprim(Juarez *et al*.,2017).

3.10.7 Molecular Detection of Integron Class1 Genes Among *Pseudomonas aeruginosa* Isolates

Class 1 Integron gene was detected after amplification with PCR technique using the primer *int*I1. The amplicon, (457bp) size was detected on agarose gel Figure (3.14).

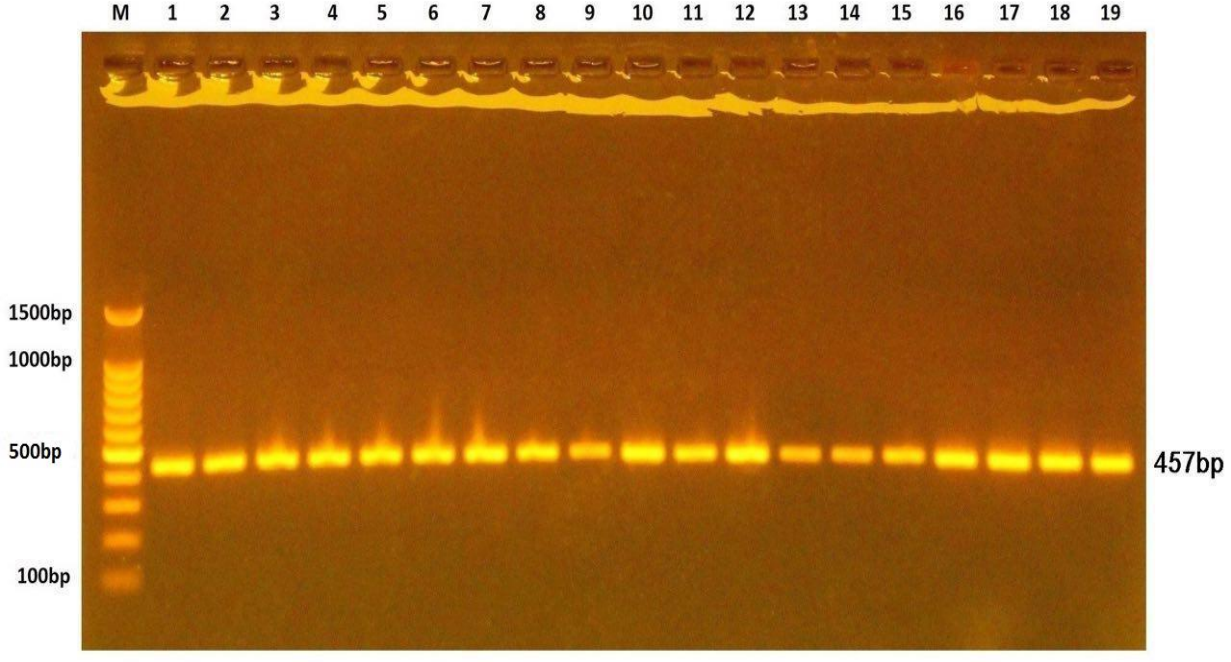


Figure (3-14): Agarose Gel electrophoresis of amplified PCR product for the detection of Intgron class1(*Int*I1) gene (457bp) run on 1.5%agarose (90 min at 70 volt) , stained with ethidium bromide, lane 1-19 *P. aeruginosa* isolates;M: Marker DNA ladder (100bp); All Lanes positive for *Int*I1 gene.

Depending on the detection of Integrase gene for the investigation of class 1 integron ,PCR assay revealed that 19 (100%) out of the 19 carbapenem resistant *P. aeruginosa* isolates were integrase positive as showen in figure (3.14), which confirm the extremely high dissemination of class 1 integron at the hospitals in Baquba / Diyala province.

This result agrees with Haghi *et al*. (2017) whose PCR results showed that 94.3% of MBL positive isolates of *P. aeruginosa* carried class 1 integron but it is different from the Iranian study presented by Ebrahimpour *et al.* (2018) that revealed 30% integron positive *P. aeruginosa* isolates and differs from study of Hsiao *et al*. (2014) who found that 41.4% of *P. aeruginosa* isolates carried this gene In southern Taiwan. The latter is close to (62.5% %) class 1 integron positive result of Opus *et al*. (2017) in Turkey. This mobile genetic element plays an important role in the dissemination of resistance genes among bacteria. It captures and integrates gene cassettes by site specific recombination and converts them to functional genes as shown by Cambray ,(2010).

The current study conducted in Diyala province showed that 16 isolates (84.21%) were positive for variable region of class 1 integron used CS primers in figure (3.15). This result disagrees with the Iraqi study conducted in Thi-Qar province by Al-Shammary, (2013) who used CS primers for amplification of the variable region of class 1 integron responsible for carbapenem resistance in *Pseudomonas* spp.which showed that only 4 out of 25 (16%) *Pseudomonas* isolates carried the mentioned gene.

Analysis of the class 1 integron variable regions revealed the presence of 4 different fragment sizes of approximately 300, 400,600 and 1500 bp figure (3-15). Nine isolates (47.36%) carried class 1 integron with sizes of approximately 1.5 kb, two isolates (10.52%) with sizes of approximately 600bp and 400 bp and three isolates (15.78%) with sizes of approximately 300bp. However, the analysis of the integron variable region revealed that 3/19 (15.78%) were lacking in gene cassettes, indicating a low occurrence of empty class 1 integrons among these strains.

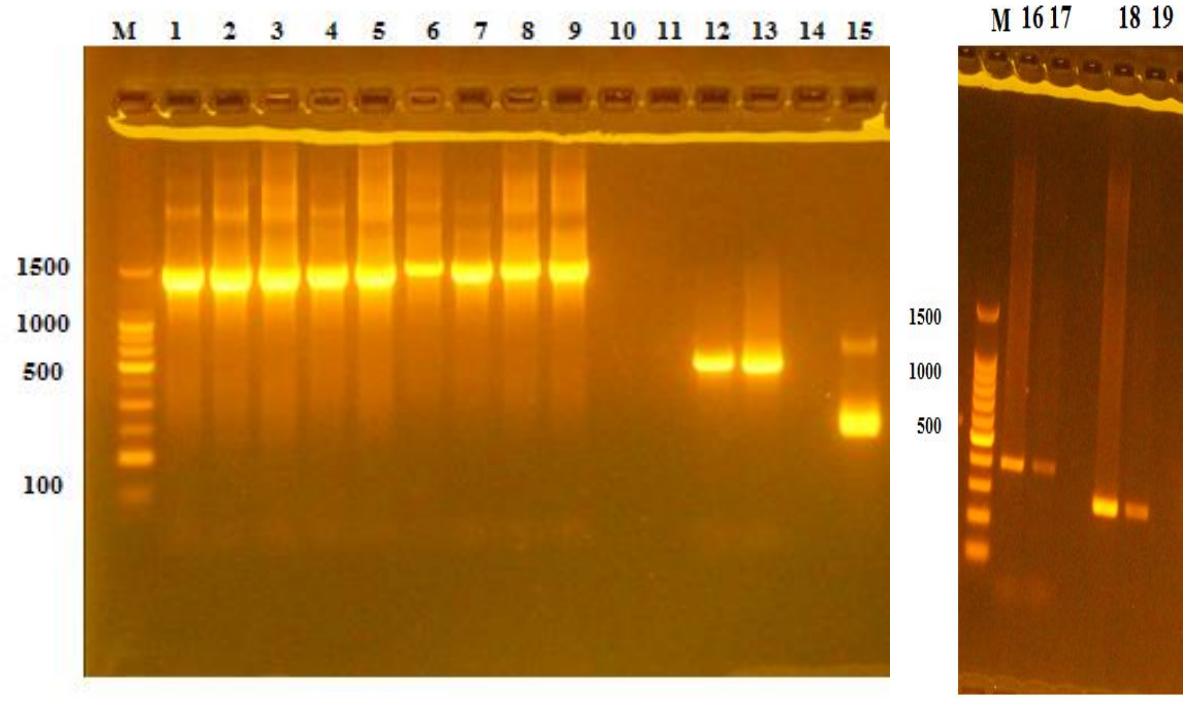


Figure (3-15): Agarose Gel electrophoresis of amplified PCR product for the detection of class1 Intgron variable regions using primersIn5'CS and In3'CS run on 1.5%agarose (90 min at 70 volt) , stained with ethidium bromide, lane 1-19 *P. aeruginosa* isolates;M: Marker DNA ladder (100bp); Lanes1,2,3,4,5,6,7, 8,9,12,13,15,16,17,18,19 positive for class1 Intgron variable regions.

Class 1 integrons have been associated with various antibiotic resistance genes and constituted an important role in the development of antimicrobial resistance. In this study, the presence of typical class 1 integrons was detected in 19 isolates (100%), indicating a high prevalence of class 1 integrons in antibiotic-resistant *P.aeruginosa* isolates in Baquba hospitals. This result is slightly higher than that recently reported from Bangladesh, in which class 1 integrons were detected in 7.14% of *P. aeruginosa* isolates collected from the hospitals in Bangladesh (Nahar *et al*.,2017). However, this result agreed with early reports of *P.aeruginosa* isolates carrying class 1 integrons, such as 91% in Tunisia (Chairat *et al*.,2019).

Class 1 integrons contribute to the emerging problem of antibiotic resistance in human medicine by acquisition, exchange, and expression of resistance genes embedded within gene cassettes (Jechalke *et al.,* 2014). Class 1 integrons frequently located in

plasmids and transposons. These have the ability to undergo horizontal transfer and contribute to rapid dissemination of antibiotic resistance genes among bacterial isolates (Fluit and Schmitz, 1999). Class 1 integrons are commonly found in *P. aeruginosa* isolated from the clinical samples as demonstrated by Zarei-Yazdeli *et al*. (2014). Therefore, the transfer of antibiotic resistance genes is often related to these integrons. Strateva and Yordanov (2018) found that MDR *P. aeruginosa* isolates have an extraordinary ability to acquire integron associated antibiotic resistance genes to adapt to their adverse environmental growth conditions in the hospital setting.

3.10.8 Clonal diversity analysis by ERIC-PCR.

Enterobacterial repetitive intergenic consensus (ERIC) sequence analysis is a powerful tool for epidemiological analysis of bacterial species. This study aimed to determine the genetic relatedness or variability in carbapenem-resistant isolates by species using this technique. In the present study, the ERIC primer sequence was used for detecting differences in the distribution and number of this bacterial repetitive sequence in the clinical isolates of *P. aeruginosa* genomes. Insilico online program were used in calculating genetic distance and obtaining a phylogenetic tree of ERIC .

Amplification of 19 *P.aeruginosa* isolates (from different specimens and location) were analyzed by ERIC-PCR generated banding pattern between 1-8 bands per isolate. Sizes ranged from (200–2000) bp, some of which (200, 300, 400, 500, 600, 900, 1000, 1300, 1500, 2000) bp were common among these isolates figure (3.16) Appendix(3).The ERIC typing of the tested isolates were recorded and subjected to computerized analysis to evaluate the genetic relations which based on the Dice coefficients .

Two main groups (**A** and **B**) were observed. ERIC-PCR typing showed 6 groups of genotypes (**a- j**) and 6 unique isolates (**b**, **d**, **e**, **g, k** and **l**), **b,e** and **l** represented **Pa5,Pa8** and **Pa9**, respectively, which were imipenem resistants. Genotype **c** was the most frequent cluster with 3 /19 (15.78%) of isolates, which were isolated from different

sources. The remaining genotypes five clusters (**a,f,h,I** and **j**) each containing 2/19 (10.52%) of isolates, from different sources as well .

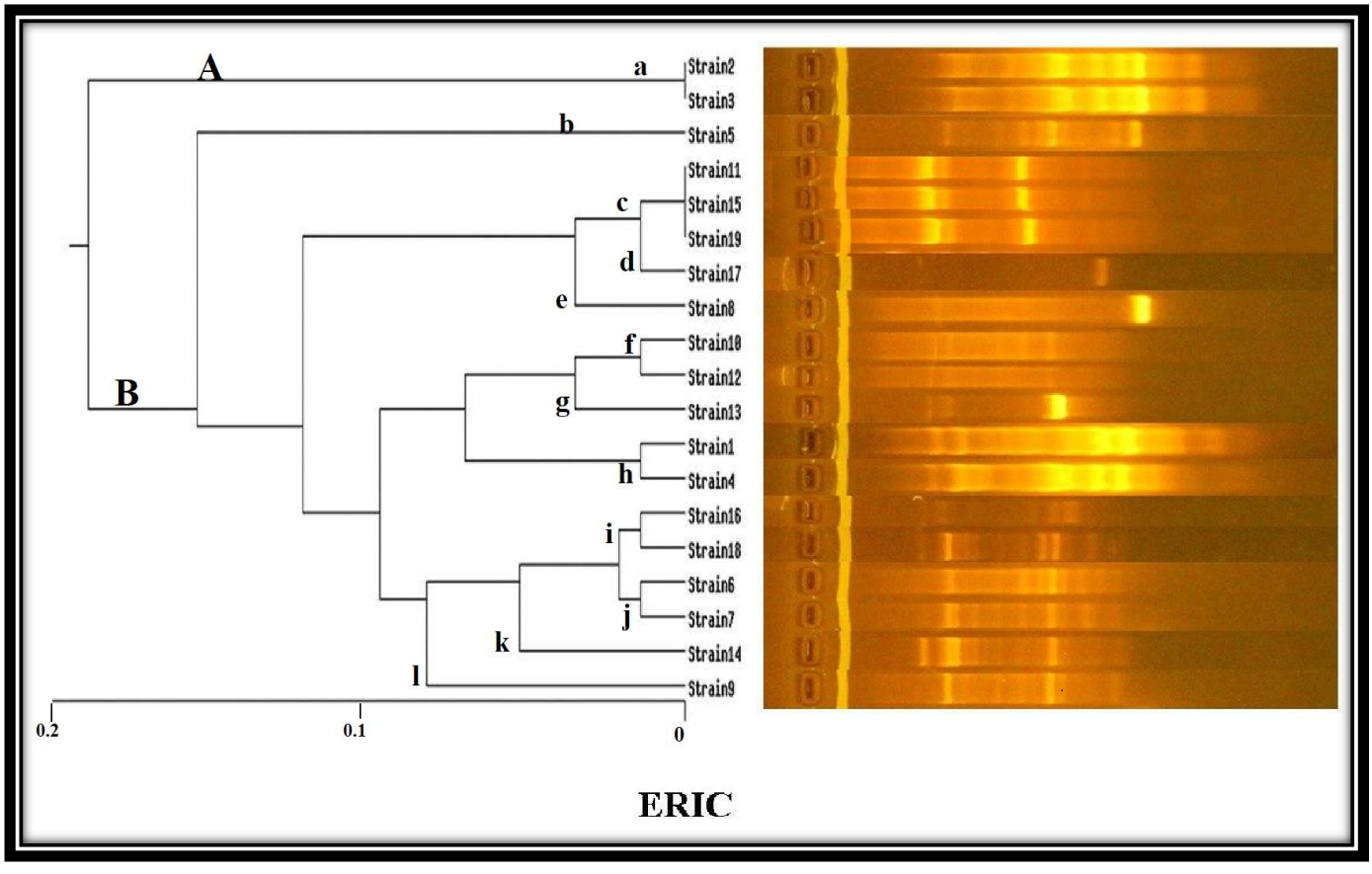


Figure (3-16). ERIC-PCR generated dendrogram showing genetic relatedness of 19 *P.aeruginosa* isolates.

In spite of differences in the location and isolation sources of these isolates, a clear clonality was observed, which indicated the epidemiology of these isolates. The sensitivity profile within the same cluster reflected a similar antibiotic resistance pattern. A similarity was observed in efflux pumps genes patterns within the same genotype, but with some exceptions like in genotype **d** which involved isolate **Pa17** was negative for *MexB, MexD and MexF*, respectively, as well as genotype **i** which included isolates **Pa16** and **Pa17** that were negative for *MexD* only (Table 3-9).

In contrast, these genotypes revealed some variability in AME genes, MBLgenes, ESBLs and Integron class 1 variable regions (Int'CS) patterns within the same genotype.

Emergence and transmission surveillance of *P.aeruginosa* strains is important among patients for infection control in a clinical setup. Various gene-typing methods have been used for epidemiological typing of *P.aeruginosa* isolates for surveillance (Auda *et al*., 2017).

High-risk-clones can easily develop antibiotic resistance through chromosomal mutations or by horizontal acquisition of resistant determinants. The increasing prevalence of multi-drug-resistant (MDR) or extensively-drug resistant (XDR) *P.aeruginosa* isolates is associated with the dissemination of these clones (Ruiz-Garbajosa and Cantón, 2017).

In addition the results from this study appear the relationship between efflux pumps genes, antibiotics resistance, XDR, PDR and ERIC-PCR, all carbapenem resistant isolates in A group contain all efflux pumps genes (expect **d** cluster have only *MexY* gene) but different in number of bands, products size, source of isolation and antibiotic resistance.

The results Sallman *et al*., (2018) showed the ERIC-PCR typing of *P. aeruginosa* bacteria that 96.82% showed amplification bands ERIC-PCR also revealed 17 groups of genotypes (A-R) and 4 unique isolates. ERIC genotyping significantly correlated resistance patterns but not with virulence control QS genes.

Infections caused by PDR/XDR are a cause of concern as they compromise the selection of appropriate empiric and definitive antimicrobial treatments. Introduction of new antibiotics with potent activity against PDR/XDR *P.aeruginosa* opens new horizons in the treatment of these infections (Ruiz-Garbajosa and Cantón, 2017).

This *P. aeruginosa* heterogeneity differences in several studies from different parts of the world, demonstrate the impact of environmental factors and the level of hospital hygiene on the distribution and genetic clonal formation variation. As the results demonstrated, our studied MDR *P. aeruginosa* clinical isolates were genetically diverse and heterogeneous, suggesting that multiple subtypes of the species are involved in

infection. Moreover the findings of present work suggest that genotyping by ERIC-PCR, may play an important role in routine epidemiological surveillance, and in the identification of the source of transmission of *P. aeruginosa* in the hospitals. As previously reported by (Khosravi *et al*.,2011), the ability of ERIC-PCR assay to discriminate types, proved to be excellent, and can be used as first screening genotyping methods for typing of *P. aeruginosa*.

Table (3-9): Correlation between ERIC- PCR typing and different drug resistance genes.

ERIC genotypes	Bands NO.	Shared bands(bp)	Efflux genes	AME genes	MBL genes	ESBL *bla*OXA10	Int'CS
a	7 bands	2000,1500,900,600,500 ,400,200	All Efflux genes	*aac(6')-Ib*(+)	*bla*VIM (+)	+	+
b	5 bands	2000,900,500,400	All Efflux genes	*aac(6')-Ib*(+)	*bla*VIM (+)	+	+
c	2 bands	2000,1000	All Efflux genes	*aac(6')-Ib*(+)	All (-)	+/-	-/+
d	One band	600	*MexY* only	*aac(6')-Ib*(+)	All (-)	-	+
e	One band	400	All Efflux genes	*aac(6')-Ib*(+)	*bla*NDM(+)	+	+
f	2 bands	1300,900	All Efflux genes	*aac(3')-II*(-)	*bla*VIM (+) *bla*NDM(+)	+	-/+
g	2 bands	1500,900	All Efflux genes	All (-)	All (-)	+	+
h	8 bands	2000, 1500,1300, 1000,900,600,500,300	All Efflux genes	*aac(6')-Ib*(+)	All (-)	-/+	+
i	4 bands	1500,1000,900,600	*MexD* (-)	*ant(4')-IIb* (-)	All (-)	_	+
j	4 bands	2000,1500,1000,600	All Efflux genes	*aac(3')-II*(-)	*bla*VIM (+) *bla*NDM(+)	+	+
K	4 bands	2000,1500,900,600	All Efflux genes	*aac(6')-Ib*(+)	All (-)	+	_
L	3 bands	1500,1000,500	All Efflux genes	*aac(6')-Ib*(+)	*bla*VIM (+)	+	+

3.10.9 Sequencing and phylogeny analysis of 16S rRNA gene

Within this locus, only one sample was included, which had shown exactly 1500 bp amplicons length of the 16S *rRNA* locus. Before sending the 16S *rRNA* amplicons to sequencing reaction, it was made sure that all the amplified amplicons had shown sharp, specific, and clean bands.

The sequencing reactions had indicated the confirmed identity of the amplified products by performing NCBI blastn (Zhang *et al*. 2000). Concerning the supposed 1500 bp PCR amplicons of the 16S *rRNA* gene, NCBI BLASTn engine has shown extremely high sequences of similarities between the sequenced samples and this target. NCBI BLASTn engine has indicated the presence of about 99% of homology with the expected target that completely covered a portion of the 16S *rRNA* gene. By comparing the observed DNA sequences of these local samples with the retrieved DNA sequences (GenBank acc. CP012901.1) the exact positions and other details of the retrieved PCR fragment were identified (Appendix 4). After positioning the 1500 bp amplicons' sequences within the 16S *rRNA* DNA sequences, the details of its sequences were highlighted Appendix (5).

The alignment results of the 1500 bp samples revealed the presence of three mutations that distributed variably in the analyzed sample in comparison with the referring 16S *rRNA* genetic sequences Appendix (6).

These 3 mutations were taken a different distribution in the analyzed samples. The sequencing chromatogram of DNA sequences as well as its detailed annotations were documented and the pattern of these variants within the amplified sequences was shown figure (3-17).

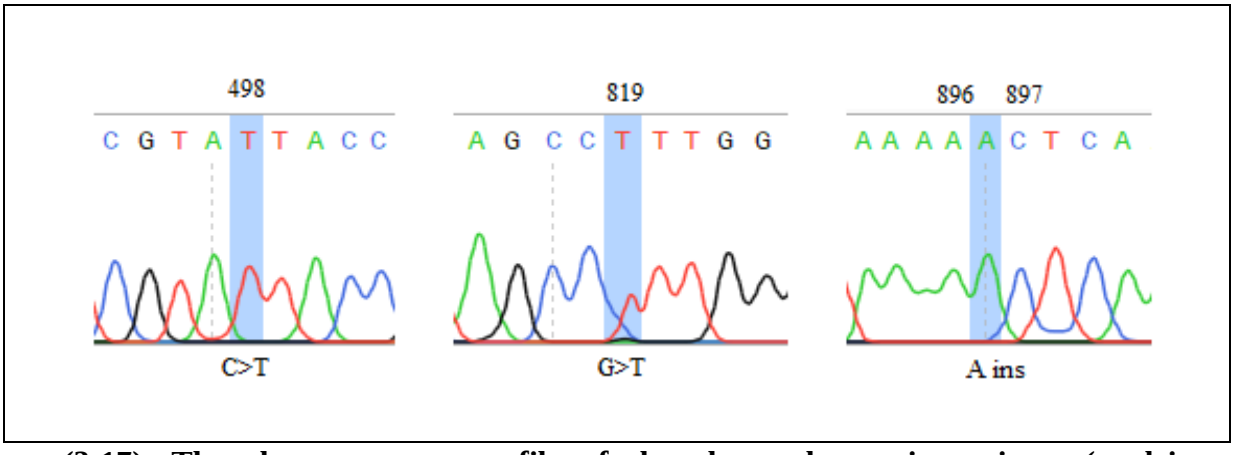


Figure (3-17). The chromatogram profile of the observed genetic variants (nucleic acids substitutions) of the 16S *rRNA* gene within *Pseudomonas aeruginosa* local isolate. Each substitution mutations highlighted according to its position in the PCR amplicon. The symbol "ins" refers to "insertion" mutation.

The observed results obtained from the sequenced 1500 bp fragment and the exact position of each observed mutation in the NCBI reference sequences are summarized in, in which the entire annotations of the observed variations were described table(3-10).

Table (3-10): The observed mutations in all of the studied specimens of 1500 bp in the *16S rRNA* DNA sequences.

No.	Reference nucleotide	Mutant nucleotide	Position in the PCR fragment	Position in the genome	Type of mutation	mutation summary
1.	C	T	498	223504	Ribosomal DNA variation	CP012901.1;g.223504C>T
2.	G	T	819	223825	Ribosomal DNA variation	CP012901.1;g.23825G>T
3.	-	A	896-897	223902-223903	Ribosomal DNA variation	CP012901.1;g.262463G>C

Subsequently, a comprehensive tree was constructed. The total number of the aligned nucleic acid sequences, irrespective of the bacterial variants, in this comprehensive tree was 99 sequences. The current constructed comprehensive tree indicated the presence of only one species all over 96 scanned of 16S *rRNA*-based variants sequence – related species, namely *P. aeruginosa* Figure (3-18). It was found

that the studied variant was located in one distinct position within this species. Despite the unique characterization of the studied 16S *rRNA* variant that originated from the currently observed variations, no deviation from *P. aeruginosa* species was noticed concerning all phylogenetically analyzed variants. Though S1(**Pa7**) bacterial isolate exhibited three nucleic acid variations no deviation was observed from other referring mates within the *P. aeruginosa* spp. Again, this fact is obviously observed in the currently constructed comprehensive phylogenetic tree as all the observed sequence-related species have belonged to *P. aeruginosa* species. This notion provided a further indication of the identity of these local studied isolates.

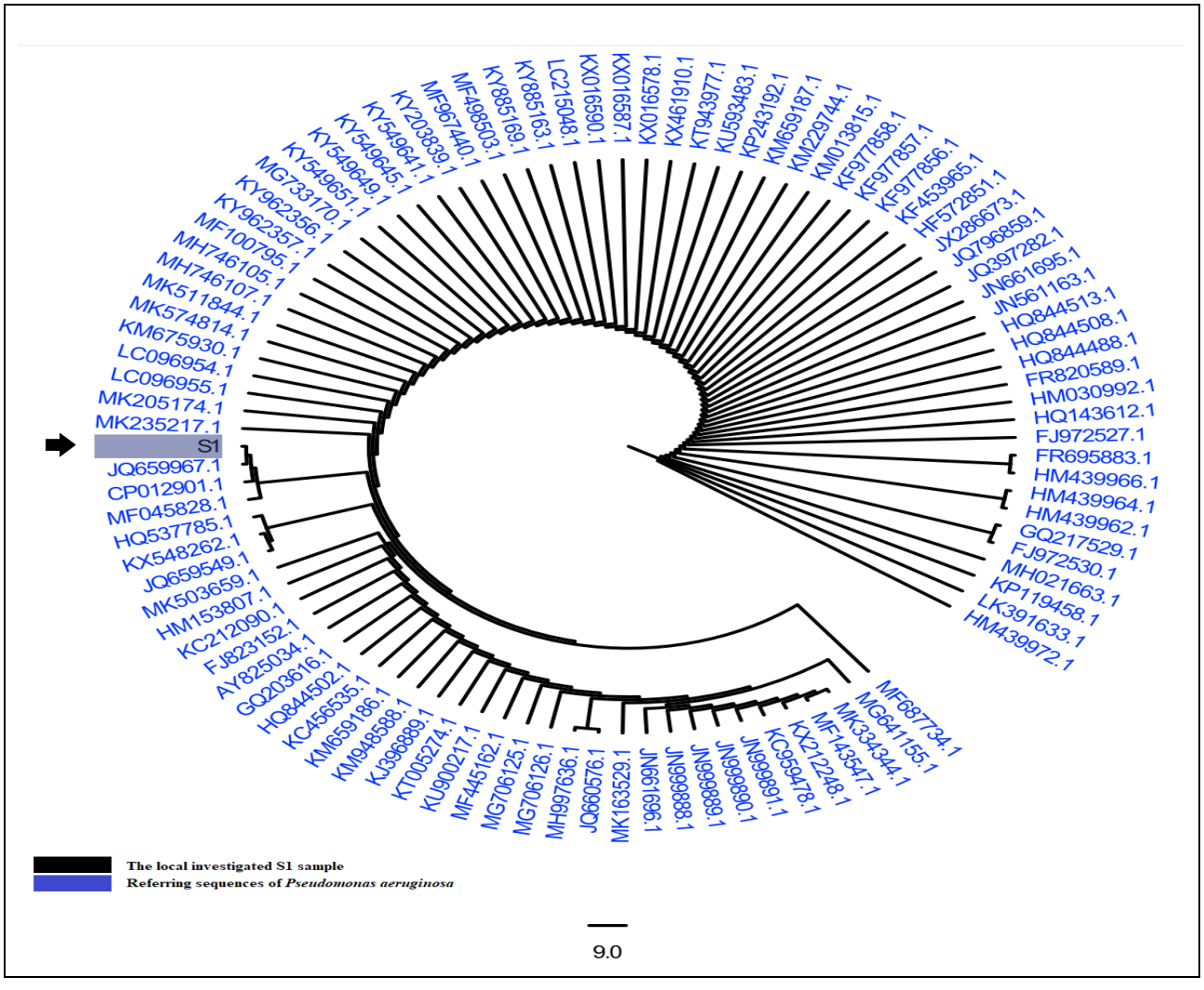


Figure (3-18) The comprehensive phylogenetic tree of genetic variants of 16S *rRNA* genetic fragment of *Pseudomonas aeruginosa* local isolate. The black color refers to the sequenced 16S *rRNA* variants, while the cyan color refers to other referring NCBI *Pseudomonas aeruginosa* deposited species. All the mentioned numbers referred to Genbank acc. no. of each referring species. The number "9.0" at the bottom of the tree refers to the degree of scale range among the comprehensive tree categorized organism s.

This 16S *rRNA*-based comprehensive tree provided an extremely inclusive tool about the high ability of such genetic fragment to efficiently identify *P. aeruginosa* bacterial isolates. Add to that, our phylogenetic analysis has observed a high *P. aeruginosa* detection specificity with regard to 16S *rRNA*- based PCR - phylogenetic protocols. In accordance with our findings, several reports have been identified an extremely high rate of efficiency in the 16S*rRNA*- based PCR-genotypic identification of *P. aeruginosa* isolates with a further ability to differentiate this species into several subspecies related groups (Alsamarraui and Alrawi 2017).

3.10.10 Recording Iraqi *P. aeruginosa* isolates in gene bank-NCBI

Two strains of *P. aeruginosa* were isolated from human sources in Baquba city/Diyala and each sequences has a symbol code (SL221814, SL8283). 16s*rRNA* gene sequences submitted to Gen Bank ,the results of these sequences were analyzed and examined by professional staff in gene bank. This strains were published in the national center for biotechnology information (NCBI) and the database of the strain was recorded in the DNA Data Bank of Japan (DDBJ) and Gene Bank for DNA sequences with accession number for six drug resistance genes. All these sequences accepted in gene bank and each sequence take accession number (MK503659, MK559695).Appendix (7) Accession number of Iraqi isolates of *P. aeruginosa* in Gene bank in table (3-11).

Of these results, the current study succeeded in relying on both isolates [Pa7(MK503659) and Pa10 (MK559695)] to be standard indicators as positive control like reference isolates to compare the results of the genetic expression of *16srRNA bla*OXA10,*bla*VIM, *aac(6')Ib, MexY, MexD and MexF* genes for other isolates based on these two isolates.

Table (3.11): Two Strain *P. aeruginosa* With Submitted ID, Name, Source, code symbol and Accession Number

Strain *P. aeruginosa*	Genes	Accession number	symbol code	Source/Region
Pa7	16S*rRNA*	MK503659	SL221814	Iraq urin isolate
Pa7	*bla*$_{OXA10}$	LC493897	SL221814	Iraq urin isolate
Pa7	*bla*$_{VIM}$	LC490649	SL221814	Iraq urin isolate
Pa7	*aac(6')Ib*	LC493895	SL221814	Iraq urin isolate
Pa7	*MexY*	LC493896	SL221814	Iraq urin isolate
Pa7	*MexD*	LC490647	SL221814	Iraq urin isolate
Pa7	*MexF*	LC490648	SL221814	Iraq urin isolate
Pa10	16S*rRNA*	MK559695	SL8283	Iraq wound isolate
Pa10	*bla*$_{OXA10}$	LC495403	SL8283	Iraq wound isolate
Pa10	*bla*$_{VIM}$	LC495404	SL8283	Iraq wound isolate
Pa10	*aac(6')Ib*	LC495320	SL8283	Iraq wound isolate
Pa10	*MexY*	LC495402	SL8283	Iraq wound isolate
Pa10	*MexD*	LC495321	SL8283	Iraq wound isolate
Pa10	*MexF*	LC495401	SL8283	Iraq wound isolate

3.11 Molecular Study of *Pseudomonas aeruginosa* RNA

3.11.1 Extraction of Total RNA

The total genomic RNA extracted from target five isolates from different clinical sources to measure the *bla*$_{OXA10}$, *aac(6')Ib* and MexY genes expression level before and after treatment with antibiotic and ZnONPs in highly precise conditions and avoid from any contamination especially RNase and the protection came from using of TRIzoL (guanidinthiocynate) with ready kit. The quantity and purity measured by Quantus Fluorometer and Nano drop spectrophotometer device which gave a range values (65-155 ng/µl) of concentration and at ratio of 260/280 the purity was ranged (1.79-1.92).

3.11.2 Gene Expression Analysis by Using Quantitative Reverse Transcriptase Real Time qRT PCR Technique (TaqMan)

The purpose of this step is to measure the expression of *bla*$_{OXA10}$, *aac(6')Ib* and *MexY* genes and compare the genes expression in the presence of antibiotics, ZnONPs and in the absence of them in order to improve the role of this genes in the resistance of *P. aeruginosa* to antibiotics.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is distinguished from other methods for gene expression due to the accuracy, sensitivity and fast results. This technology is the golden standard for gene expression analysis. It is important to realize that in a relative quantification study, the experiments are usually interested in comparing the expression level of a particular gene among different samples (Derveaux *et al*., 2010).

Real time PCR quantification applied in this study utilizes the TaqMan qPCR. Amplification of fragment of mRNA was performed with the following master amplification reaction with the program of One-Step RT-PCR. Species-specific primers and probes were designed for *bla*$_{OXA10}$, *aac(6')Ib and MexY* genes as an identification target of *P. aeruginosa* and a reference gene for evaluation of selected virulence genes expression.The amplifications were recorded as Ct value (cycle threshold). The house keeping gene used in the study was *rpsL* gene(201bp).Using this gene in molecular studies ensures that expression remains constant in the cells or tissues under investigation and different conditions (Reboucas *et al*., 2013). This gene was used to normalize the mRNA levels of genes of interest before the comparison between different samples by the real time PCR. The experiment of quantitative PCR reaction was done by using 5 resistant isolates of *P. aeruginosa* which had *bla*$_{OXA10}$, *aac(6')Ib and MexY* genes. These isolates were chosen with sub MIC values to antibiotic and ZnONPs.

No information was locally formed regarding the role of zinc oxide nanoparticles ZnO NPs in *bla*$_{OXA10}$, *aac(6')Ib and MexY* gene expression in this bacterium. Therefore,

this study focused on the role of these three drug resistance associated genes and on the effect of the ZnO NPs on *bla$_{OXA10}$, aac(6')Ib and MexY* gene expression.

3.11.3 Real time PCR Quantification of *bla*$_{OXA10}$ GeneExpression

The experiment of the quantitative RT-PCR reaction was completed by using five (5) from (19) of *P. aeruginosa* isolates. The different sources of these isolates were distributed as follows (1 wound, 1 burn, 1 urin, 1 sputum and 1 ear). They a *bla$_{OXA10}$* gene and which gave a highly expression of different degrees. This variation refers to the source of isolates. The Ct value of *bla*$_{OXA10}$ gene in the present study is shown in Table (3-13) and Appendix (6) that show the pattern of the amplification of the gene.

The expression was compared with *rpsl* gene expression to measure the gene expression of the *bla*$_{OXA10}$ genes and compare it with the housekeeping gene. The assay was performed three times for each sample and the mean of three values considered as quantity of given gene expression for that sample. The ΔΔ Ct was used for determinining gene expression. The ΔΔ Ct was gained by subtracting Δ Ct of sample from Δ Ct of reference gene.

There were significant differences according to χ^2 values at P<0.05 found between the isolates. These differences depend on the range of Ct value from (11.64 to 19.94). This result was achieved before treating the isolates with ceftazidime . The results were presented when the isolates were treated with ceftazidime using a concentration that was below the dose of sub MIC for each sample. The range of Ct value for the gene under this study was from (11.40 to 18.25), as represent in Table (3-12). The results were presented when the isolates were treated with ZnO NPs using a concentration that was below the dose of sub MIC for each sample. The range of Ct value for the gene under this study was from (12.15to 18.93). Besides the pattern of the amplification of the gene is shown in Figure (3-19) and Appendix (9) respectively.

Table (3-12): The Ct Value of *bla*$_{OXA-10}$ gene before and after Treatment with Ceftazidime and ZnONPs.

No. of isolates n=5	Source of isolates	Ct value of *bla*$_{OXA10}$ before treated	Ct value of *bla*$_{OXA10}$ after treated with CAZ	χ^2 (P-value)	Ct value of *bla*$_{OXA10}$ after treated with ZnONP	χ^2 (P-value)
Pa2	Burn	11.64	12.52	3.28*(0.061)	13.99	3.9*(0.04)
Pa5	Ear	12.19	11.63	0.72*(0.427)	12.15	0.1 (0.9)
Pa7	Urin	13.74	11.40	0.47*(0.571)	12.47	0.63*(0.47)
Pa8	Sputum	14.22	12.87	0.38*(0.627)	16.47	0.61*(0.512)
Pa10	Wound	19.94	18.25	0.74*(0.411)	18.93	3.9* (0.042)
*** P<0.05**						

The expression of *bla*$_{OXA10}$ showed highest level of folding with the average of 4.06 in the case of isolates that treated with ceftazidime at the concentration of 128µg/ml.Three isolates (Pa5,Pa8 and Pa10) showed overexpression of *bla*$_{OXA10}$ with higher MIC of ceftazidime (256-512 µg/ml) . The sub-MIC of ZnO nanoparticles effectiveness on expression of both *bla*$_{OXA10}$ and *rpsĹ* genes in selected target isolates are illustrated below in Tables (3-12) and (3-13), shown there were a significant differences according to χ^2 values at p<0.05 among target isolates in Mean Δ C_T**s**(Treated), Mean Δ C_T**c** (Untreated), **Δ Δ** C_T, and $2^{-(\Delta\Delta CT)}$ fold change of qRT-PCR resultes.

Table (3-13): Ct values and fold of gene expression of *bla*$_{OXA10}$ and *rpsL* genes of *P. aeruginosa* that was treated with Ceftazidime and ZnONP.

Groups	No. of isolates	Mean Ct of refrence *rpsL*	Mean Ct of target *bla*$_{OXA10}$	ΔCt	ΔΔCt	(Folding)= $2^{-\Delta\Delta Ct}$	Avarage of folding
Untreated	Pa2	16.61	11.64	-4.98	0.00	1.00	**1**
	Pa5	16.37	12.19	-4.18	0.00	1.00	
	Pa7	19.28	13.74	-5.54	0.00	1.00	
	Pa8	30.21	14.22	-15.99	0.00	1.00	
	Pa10	16.95	19.94	2.99	0.00	1.00	
Treated with CAZ (128µg/ml)	Pa2	18.83	12.52	-6.32	-1.34	2.53	**4.06**
	Pa5	18.13	11.63	-6.50	-2.32	5.00	
	Pa7	17.01	11.40	-5.61	-1.18	2.27	
	Pa8	18.56	12.87	-5.69	-0.16	6.12	
	Pa10	17.40	18.25	0.85	-2.13	4.39	
Treated with ZnO (325µg/ml)	Pa2	16.03	13.99	-2.04	2.93	0.13	**0.45**
	Pa5	16.97	12.15	-4.82	-0.65	0.56	
	Pa7	17.04	12.47	-4.58	0.96	0.52	
	Pa8	17.44	16.47	-0.97	15.02	0.001	
	Pa10	16.00	18.93	2.93	-0.05	1.04	

CAZ = Ceftazidime

Treated with ZnO NPs that led to the decrease in the value of gene expression in four isolate of *bla*$_{OXA10}$ that was nearly average (0.45) at the concentration of 325µg/ml table

(3-13) range changed of 0.001 to 0.13-fold in *bla*$_{OXA10}$ gene expression due to effectiveness exposure of 325µg/ml ZnO NPs which highly effected on *bla*$_{OXA10}$ expression. On the other hand the isolate (Pa10) showed no affected due to ZnO NPs sub-MIC that the fold values were 1.04 also there were no changing in expression folds no decreasing, nor increasing and stayed in range of one (1) .

Regarding to effect of metal nanoparticles ZnO on microorganisms DNA replication and gene expression take a diversified potentiality ranged, from highest pivotal changing may up to stopped genetic expression for targeted genes, to marginal or impalpable functionally illustrated through of Sharma *et al*., (2009).

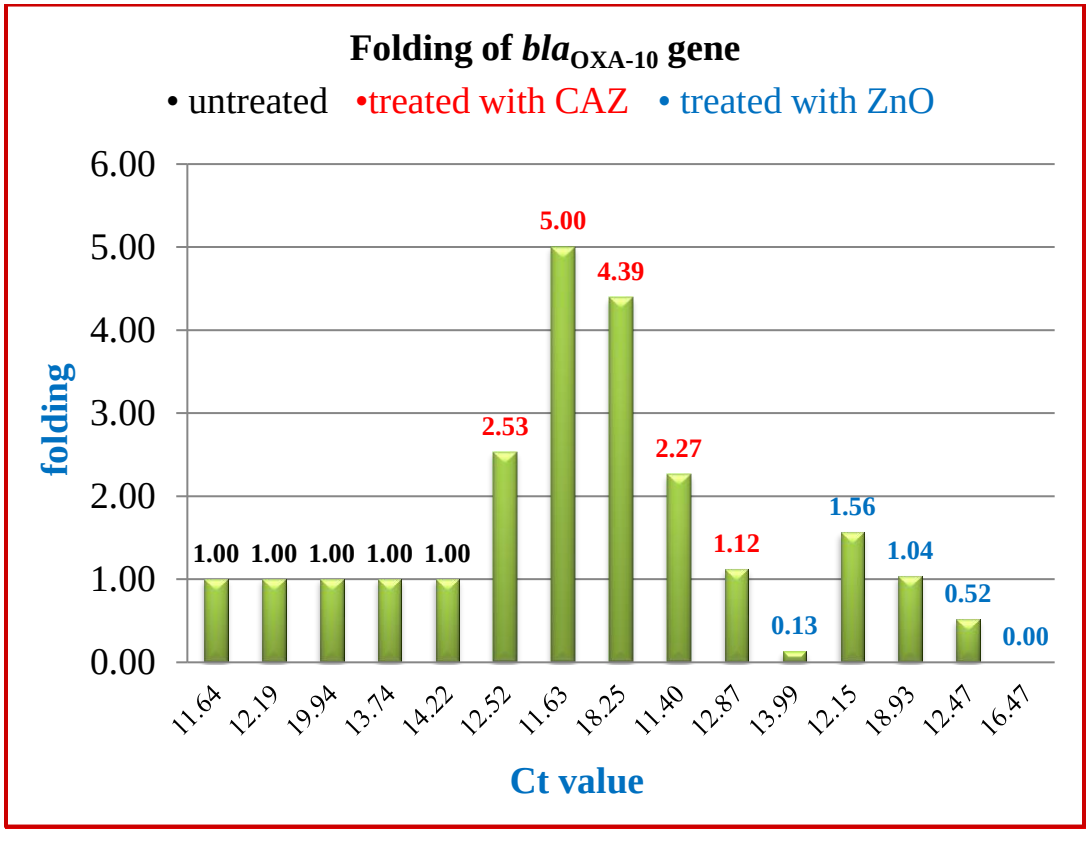


Figure (3-19): Folding change of *bla*$_{OXA10}$ gene expression

Although there are no studies on the effect of ZnO nanoparticles on the gene expression of ESBLs *bla*$_{OXA10}$ of *P. aeruginosa*, but it should be mentioned that there are other studies targeted other genes in this bacteria by other antimicrobial agents. The other studies focused about restriction of growth of pathogenic bacteria by either

antibiotics or other innovative antimicrobial agents like nanoparticles, without addressing and studying the effectiveness of nanoparticles and antibiotics on gene expression of ESBLs encoding genes. Therefore the present study could be the newest one in all its recent contents regarding of *P. aeruginosa* ESBLs enzymes restriction.

3.11.4 Real time PCR Quantification of *aac(6')Ib* Gene Expression

The experiment of the quantitative RT-PCR reaction was completed by using five (5) isolates of *P. aeruginosa* from different sources .They have a *aac(6')Ib* gene and which gave a highly expression of different degrees. The Ct value of *aac(6')Ib* gene in the present study is shown in Table (3-14) and Appendix (10) that show the pattern of the amplification of the gene.

The expression was compared with *rpsl* gene expression to measure the gene expression of the *aac(6')Ib* gene and compare with the housekeeping gene. The assay was performed three times for each sample and the mean of three values considered as quantity of given gene expression for that sample. The ΔΔ Ct was used for determine gene expression. The ΔΔ Ct was gained by subtracting Δ Ct of sample from Δ Ct of reference gene.

There were significant differences according to χ^2 values at $P<0.05$ found between the isolates. These differences depend on the range of Ct value from (11.39 to 28.17). This result was before treated with gentamicin . The results were presented when the isolates were treated with gentamicin using a concentration that was below the dose of sub MIC for each sample. The range of Ct value for the gene under this study was from (11.88 to 24.31), as represent in Table (3-14).

Table (3-14): The Ct Value of *aac(6')Ib* gene before and after Treatment with Gentamicin and ZnONP.

No. of isolates n=5	Source of isolates	Ct value of *aac(6')Ib* before treated	Ct value of *aac(6')Ib* after treated with CN	χ^2 (P-value)	Ct value of *aac(6')Ib* after treated with ZnONP	χ^2 (P-value)
Pa2	Burn	11.39	11.88	2.84***(**0.073)	12.00	3.26*****(0.068)
Pa5	Ear	15.56	12.82	0.42*****(0.542)	12.66	1.88*****(0.27)
Pa7	Urin	16.19	14.27	0.61 (0.445)	12.60	2.41*****(0.123)
Pa8	Sputum	15.57	16.11	2.362*****(0.117)	17.59	2.09*****(0.207)
Pa10	Wound	28.17	24.31	3.27***** (0.067)	27.89	4.3***** (0.034)
*** P<0.05**						

CN = Gentamicin

The results were presented when the isolates were treated with ZnO NPs using a concentration that was below the dose of sub MIC for each sample. The range of Ct value for the gene under this study was from (12 to 27.89). Besides the pattern of the amplification of the gene is shown in Figure (3-20) and Appendix (10) respectively.

The expression of *aac(6')Ib* showed highest level of folding with the average of **14.77** in the case of isolates that treated with gentamicin at the concentration of 256µg/ml.Two isolates (Pa5 and Pa10) showed overexpression of *aac(6')Ib* with fold change 30.22 and 32.08 respectively.All isolates show high level of MIC of gentamicin (512-1024 µg/ml) . The sub-MIC of ZnO nanoparticles effectiveness on expression of both *aac(6')Ib* and *rpsĹ* genes in selected target isolates are illustrated below in Tables (3-14) and (3-15), shown there were a significant differences according to χ^2 values at $p<0.05$ among target isolates.Treated with ZnO NPs that led to the decrease in the value of gene expression in three isolates of *aac(6')Ib* that was 0.001,0.44 and 0.63 at the

concentration of 325µg/ml table (3-14). This due to effectiveness exposure of 325µg/ml ZnO NPs which highly effected on *aac(6')Ib* expression. On the other hand the isolates (Pa5 and Pa7) showed no affected due to ZnO NPs sub-MIC that the fold values were 2.56 and 11.36 also there were increasing in expression folds and not effected by ZnO NPs.

Table (3-15): Ct values and fold of gene expression of *aac(6')Ib* and *rpsL* genes of *P. aeruginosa* that was treated with Gentamicin and ZnONP.

Groups	No. of isolates	Mean Ct of refrence *rpsL*	Mean Ct of target *aac(6')Ib*	ΔCt	ΔΔCt	(Folding)= $2^{-\Delta\Delta Ct}$	Avarage of folding
Untreated	Pa2	16.61	11.39	-5.23	0.00	1.00	**1**
	Pa5	16.37	15.56	-0.81	0.00	1.00	
	Pa7	19.28	16.19	-3.09	0.00	1.00	
	Pa8	30.21	15.57	-14.64	0.00	1.00	
	Pa10	16.95	28.17	11.22	0.00	1.00	
Treated with CN (256µg/ml)	Pa2	17.58	11.88	-5.71	-0.48	1.40	**14.77**
	Pa5	18.55	12.82	-5.73	-4.92	30.22	
	Pa7	20.51	14.27	-6.24	-3.15	8.89	
	Pa8	31.06	16.11	-14.95	-0.32	1.24	
	Pa10	18.09	24.31	6.22	-5.00	32.08	
Treated with ZnO (325µg/ml)	Pa2	16.03	12.00	-4.03	1.20	0.44	**3.00**
	Pa5	16.97	12.66	-4.31	-3.51	11.36	
	Pa7	17.04	12.60	-4.45	-1.36	2.56	
	Pa8	17.44	17.59	0.15	14.79	0.001	
	Pa10	16.00	27.89	11.89	0.67	0.63	

CN = Gentamicin

Although there are no studies on the effect of ZnO nanoparticles and gentamicin on the gene expression of Aminoglycoside-Modifying Enzyme (AME) *aac(6')Ib* of *P. aeruginosa*, but it should be mentioned that there are other studies targeted other genes in this bacteria by other antimicrobial agents. Therefore the present study could be the first study of *P. aeruginosa aac(6')Ib* gene expression.

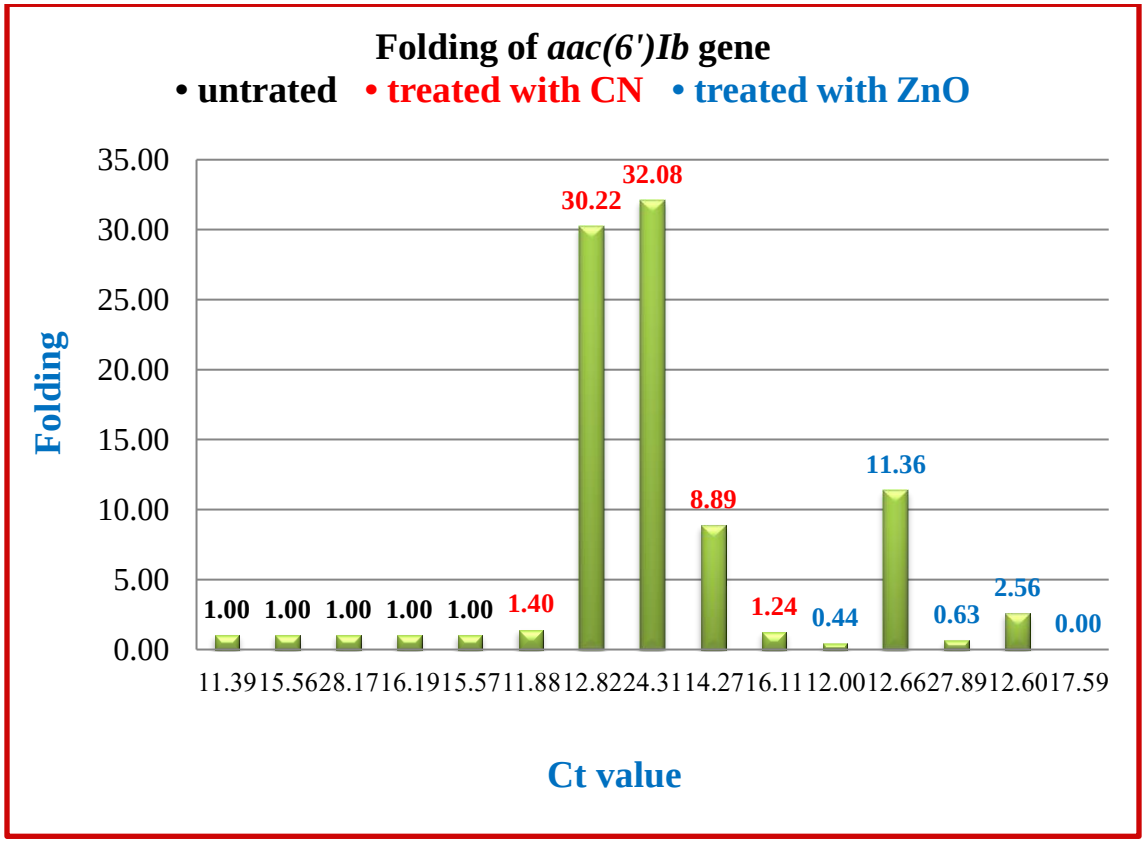


Figure (3-20): Folding change of *aac(6')Ib* gene expression

3.11.5 Real time PCR Quantification of *MexY* Gene Expression

The experiment of the quantitative RT-PCR reaction was completed by using five (5) isolates of *P. aeruginosa* from different sources .They have a *MexY* gene and which gave a highly expression of different degrees. The Ct value of *MexY* gene in the present study is shown in Table (3-16) and Appendix (11) that show the pattern of the amplification of the gene.

There were significant differences according to χ^2 values at P<0.05 found between the isolates. There differences depend on the range of Ct value from (18.50 to 21.54 before treated with gentamicin. The results were presented when the isolates were treated with gentamicin using a concentration that was below the dose of sub MIC for each sample. The range of Ct value for the gene under this study was from (17.84 to 19.88), as represent in Table (3-16).

Table (3-16): The Ct Value of *MexY* gene before and after Treatment with Gentamicin and ZnONPs.

No. of isolates n=5	Source of isolates	Ct value of *MexY* gene before treated	Ct value of *MexY gene* after treated with CN	χ^2 (P-value)	Ct value of *MexY gene* after treated with ZnONP	χ^2 (P-value)
Pa2	Burn	21.54	17.84	0.11(0.765)	21.58	0.09 (0.977)
Pa5	Ear	18.50	18.60	0.09 (0.984)	20.02	3.8* (0.050)
Pa7	Urin	20.90	19.88	0.08 (0.937)	19.43	3.8* (0.050)
Pa8	Sputum	20.59	19.30	0.41*(0.63)	20.69	0.46*(0.574)
Pa10	Wound	19.04	19.28	0.28 (0.721)	20.69	0.09(0.987)
*** P<0.05**						

CN= Gentamicin

The results were presented when the isolates were treated with ZnO NPs using a concentration that was below the dose of sub MIC for each sample. The range of Ct value for the gene under this study was from (19.43 to 21.58) . Besides the pattern of the amplification of the gene is shown in Figure (3-21) and Appendix(8) respectively.

The expression of *MexY* showed highest level of folding with the average of **8.16** in the case of isolates that treated with gentamicin at the concentration of 256µg/ml.One isolate (Pa2) showed overexpression of *MexY* with fold change 25.52 .All isolates show

high level of MIC of gentamicin (512-1024 µg/ml) . The sub-MIC of ZnO nanoparticles effectiveness on expression of both *MexY* and *rpsĹ* genes in selected target isolates are illustrated below in Tables (3-16) and (3-17). Treated with ZnO NPs that led to the decrease in the value of gene expression in all five isolates of *MexY* with avarege 0.39 at the concentration of 325µg/ml. This due to effectiveness exposure of 325µg/ml ZnO NPs which highly effected on *MexY* expression.

Efflux-mediated resistance has been found in many bacterial genera. Over expression of an efflux system, responsible for reduction in the accumulation of the antibiotic . The Mex efflux pumps of *P. aeruginosa* are of particular interest because of their exceptionally broad substrate specificity. While 12 potential efflux systems of this family have been identified in the *P. aeruginosa* genome(Mesaros *et al*.,2017) .Four of them (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM) are best characterized as antibiotic transporters (Li *et al*. ,2016). Multidrug pumps, particularly those represented by the clinically relevant AcrAB-TolC and Mex pumps of the resistance-nodulation-division (RND) superfamily, not only mediate intrinsic and acquired multidrug resistance (MDR) but also are involved in other functions, including the bacterial stress response and pathogenicity. Additionally, efflux pumps interact synergistically with other resistance mechanisms (e.g., with the outer membrane permeability barrier) to increase resistance levels (Li *et al*.,2015).

Table (3-17): Ct values and fold of gene expression of *MexY* and *rpsL* genes of *P. aeruginosa* that was treated with Gentamicin and ZnONP.

Groups	No. of isolates	Mean Ct of refrence *rpsL*	Mean Ct of target *MexY*	ΔCt	ΔΔCt	(Folding)= $2^{-\Delta\Delta Ct}$	Avarage of folding
Untreated	Pa2	16.61	21.54	4.92	0.00	1.00	**1**
	Pa5	16.37	18.50	2.14	0.00	1.00	
	Pa7	19.28	20.90	1.62	0.00	1.00	
	Pa8	30.21	20.59	-9.62	0.00	1.00	
	Pa10	16.95	19.04	2.09	0.00	1.00	
Treated with CN	Pa2	17.58	17.84	0.25	-4.67	25.52	**8.16**
	Pa5	18.55	18.60	0.05	-2.09	4.26	
	Pa7	20.51	19.88	-0.63	-2.25	4.76	
	Pa8	31.06	19.30	-11.76	-2.14	4.42	
	Pa10	18.09	19.28	1.19	-0.90	1.87	
Treated with ZnO (325µg/ml)	Pa2	16.03	21.58	5.55	0.63	0.65	**0.39**
	Pa5	16.97	20.02	3.05	0.92	0.53	
	Pa7	17.04	19.43	2.38	0.76	0.59	
	Pa8	17.44	20.69	3.25	12.87	0.001	
	Pa10	16.00	20.69	4.69	2.60	0.16	

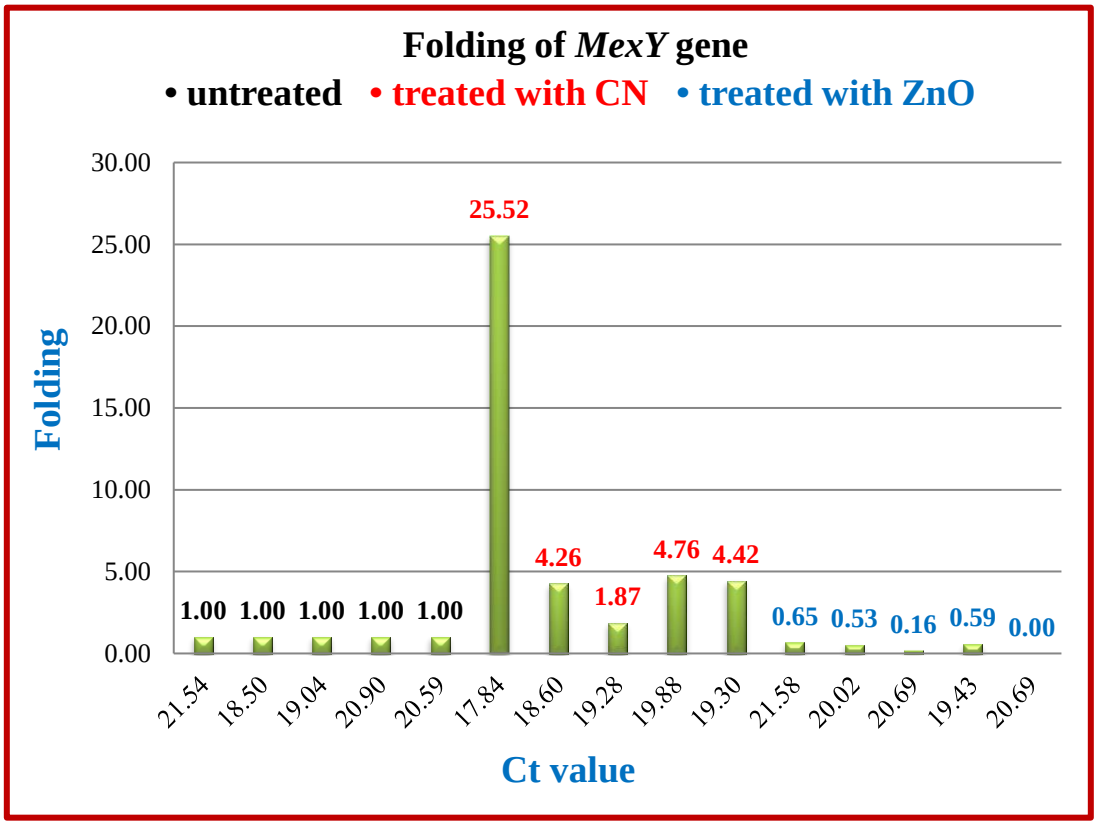


Figure (3-21): Folding change of *MexY* gene expression

The killing mechanism of nano zinc oxide indicates to different issues, where the correct toxicity system is not very clear and still controversial. There are also a few inquiries into the activity against bacteria that require deep explanations. The specific and recorded killing mechanisms are as follows: direct contact of ZnO-NPs with cell walls, or destruction of bacterial cells (Jiang *et al*., 2018), or the release of Zn ions as antibodies or the formation of effective oxygen ions (Ali and Thalij, 2017).

Table (3-19) shows the MICs and gene expression levels of resistant isolates. All 5 isolates demonstrated different levels of the resistance mechanisms. Based on the overexpression of bla_{OXA10} , *aac(6')Ib* and *MexY* .

Table (3-18): MIC and Gene expression levels of high level carbapenem- resistant (XDR&PDR) target *P. aeruginosa* isolates.

Strain	MIC (µg/ml)			Gene expression					
				bla_{OXA10}		*aac(6')Ib*		*MexY*	
	CAZ	CN	IMP	Treat with CAZ	Treat with ZnO	Treat with CN	Treat with ZnO	Treat with CN	Treat with ZnO
Pa2	256	512	128	2.53	0.13	1.40	0.44	**25.52**	0.65
Pa5	512	1024	32	**5.00**	0.56	**30.22**	11.36	4.26	0.53
Pa7	512	512	128	2.27	0.52	**8.89**	2.56	4.76	0.59
Pa8	512	1024	128	**6.12**	0.001	1.24	0.001	4.42	0.00
Pa10	256	512	256	4.39	1.04	**32.08**	0.63	1.87	0.16

Conclusions

1- *Pseudomonas aeruginosa* was recognized as a major public health problem and the misuse of antibiotics led to increasing emergence of *P. aeruginosa* resistant isolates (MDR, XDR and PDR).

2- The prevalence of increasing resistance rate to Carbapenems, the final drug choice for the treatment of *P. aeruginosa*, among patients is a threatening matter due to the increasing usage of this group especially Meropenem.

3- Most of Carbapenem resistant (XDR and PDR) *P.aeruginosa* were carrying more than one of four clinically important efflux pumps genes.

4- Molecular technique (PCR), provided sensitive and rapid analytical tools for identification and sequencing, it also increases the excellence of epidemiological investigations.

5- The extensively high dissemination of class1Integron gene among *P.aeruginosa* revealed by this study is considered dangerous due to the responsibility of this gene in antibiotic resistance dissemination among this bacterial population or to other genera by horizontal transmission predicting epidemiology.

6- All the target isolates demonstrated different levels of resistance mechanisms based on the overexpression of bla_{OXA10}, *aac(6')Ib* and *MexY* genes after treatment with antibiotics.

7- The capability of ZnO nanoparticles to downregulate the expression of resistance genes of *P. aeruginosa* was at MIC 325µg/ml. The decrease in bla_{OXA10}, *aac(6')Ib* and *MexY* genes expression was in different folds.

8- The developed primer pair and hydrolyzed probe has adequate resolution power for specific identification of *P. aeruginosa* and can be used as a valid, rapid and non-expensive alternative for conventional methods for identification and enumeration of *P. aeruginosa* in clinical samples.

Recommendations:

1- Studying gene expression of other classes of efflux pumps genes to be carried out in the local isolates of *Pseudomonas aeruginosa* in Iraqi hospitals.
2- Comparative genomic analysis and identification of pathogenicity islands of hypervirulent *Pseudomonas aruginosa*.
3- Studying distribution, function and regulation of type III and type 6 secretion systems of *Pseudomonas aruginosa*.
4- In the way to face antibiotic era failure, it is important to adopt a new type of studies about using bacteriophages in the treatment of bacterial infections trying to apply these modern drugs in Iraqi hospitals.
5- Conducting further molecular studies to detect the changes in the patterns of the local bacteria by modern molecular methods and using the MLST in the analysis of other pathogenic bacteria to certify its classification and diversity.

A

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Appendix (1): Chart Report of VITEK-2 System for identification the Results of *Pseudomonas aeruginosa*

مختبر الشمس التخصصي

bioMérieux Customer: هادي علي

Microbiology Chart Report

Printed Apr 3, 2018 16:15 CDT

Patient Name: Lina Abdil Ameer, 1
Location:
Lab ID: 55

Patient ID: aa55
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source: Unknown

Collected:

Comments:	

Identification Information	**Analysis Time:**	4.83 hours	**Status:**	Final
Selected Organism	98% Probability **Bionumber:**	**Pseudomonas aeruginosa** 0043043303500240		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	+	13	dGLU	+	14	GGT	+	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	-	20	dMNE	-	21	BXYL	-	22	BAlap	+
23	ProA	+	26	LIP	+	27	PLE	-	29	TyrA	+	31	URE	+	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	+	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	+	57	BGUR	-
58	O129R	-	59	GGAA	-	61	IMLTa	+	62	ELLM	-	64	ILATa	-			

Appendix

مختبر الشمس التخصصي

bioMérieux Customer: هادي علي

Microbiology Chart Report

Printed May 11, 2018 19:32 CDT

Patient Name: Ibrahim Salih, .
Location:
Lab ID: 137

Patient ID: aa137
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source: Urine

Collected:

Comments:	

Susceptibility Information	**Analysis Time:** 13.88 hours			**Status:** Final	
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Piperacillin/Tazobactam	64	S	Amikacin	>= 64	R
Ceftazidime	>= 64	R	Gentamicin	8	I
Cefepime	32	R	Tobramycin	>= 16	R
Imipenem	>= 16	R	Ciprofloxacin	>= 4	R
Meropenem	8	R	Levofloxacin	>= 8	R

+= Deduced drug *= AES modified **= User modified

AES Findings		
Confidence:	Consistent	
Phenotypes flagged for review:	BETA-LACTAMS	ESBL + R CARBAPENEMS (IMPER),HL CASE + R CARBAPENEMS (IMPER)

Appendix (2) Antimicrobial agents used in the current study(CLSI,2018).

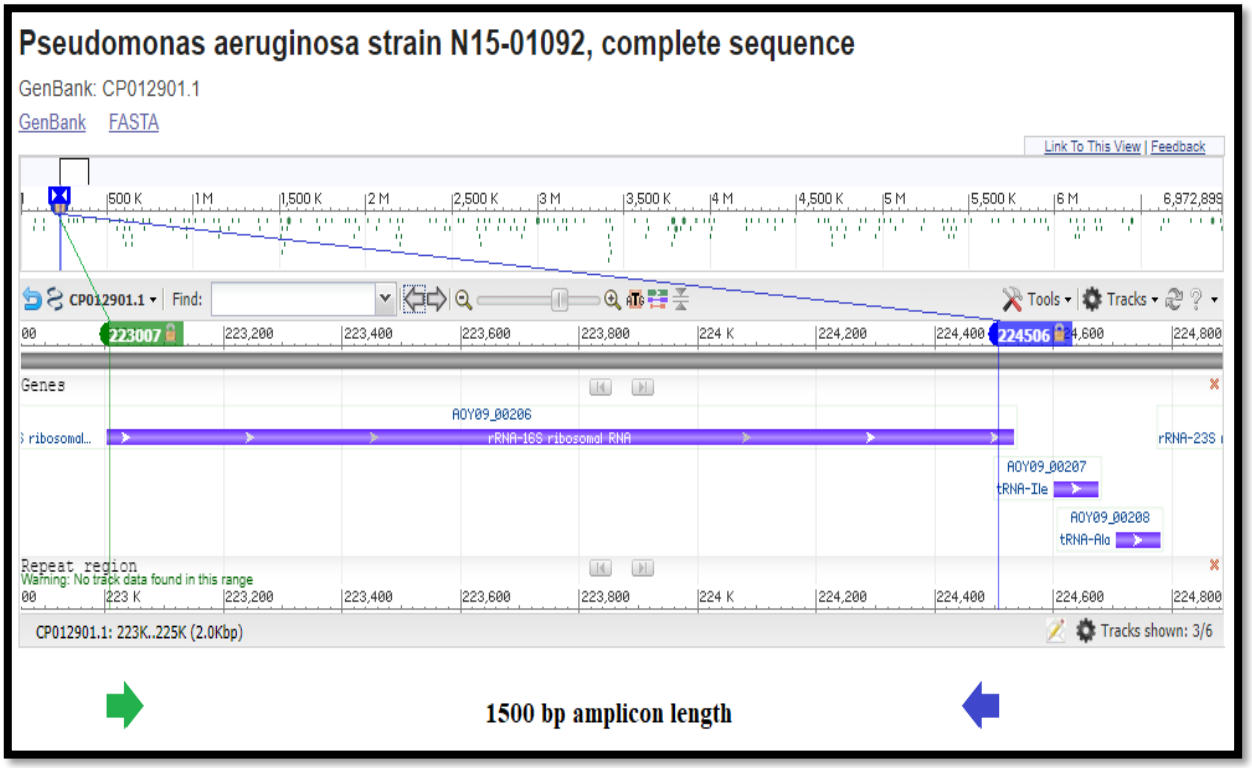
Id	Antimicrobial agent	Disc potency (µg /Disc)	Diameter of zone inhibition (mm)		
			Resistant	Intermediate	Sensitive
1	Pipracilin	100	≤ 14	15-20	≥21
2	Ticarcillin	30	≤ 14	15-20	≥20
3	Ticarcillin/clavulanic acid	75/10	≤15	16-23	≥24
4	Amoxicillin/Clavulanic acid	20/10	≤ 14	-	≥20
5	Cefotaxime	30	≤14	16-18	≥18
6	Ceftriaxone	10	≤14	15-18	≥18
7	Ceftazidime	30	≤14	15-17	≥18
8	Cefepime	30	≤14	15-17	≥18
9	Ciprofloxacin	5	≤15	16-20	≥21
10	Levofloxacin	5	≤13	14-16	≥17
11	Oflaxacin	5	≤12	13-15	≥16
12	Gentamicin	10	≤12	13-14	≥15
13	Amikacin	30	≤ 14	15-16	≥ 17
14	Tobramycin	10	≤12	13-14	≥15
15	Streptomycin	10	≤12	13-14	≥15
16	Aztreonam	30	≤ 15	16-21	≥ 22
17	Imipenem	10	≤15	16-18	≥19
18	Meropenem	10	≤15	16-18	≥19

Appendix (3) ERIC analysis

Strain1;1;1;1;1;1;1;0;1
Strain2;0;1;1;1;1;1;1;1;
Strain3;0;1;1;1;1;1;1;1;
Strain4;1;1;1;1;1;1;0;1
Strain5;0;1;0;1;1;1;1;0
Strain6;0;1;0;1;1;0;1;0
Strain7;0;1;0;1;1;0;1;0
Strain8;0;0;0;0;0;0;1;0
Strain9;0;1;0;0;0;1;0;1
Strain10;0;0;0;1;1;0;0;0;
Strain11;0;0;0;1;0;0;0;1;
Strain12;0;0;0;1;1;0;0;0;
Strain13;0;0;1;0;0;1;0;0;
Strain14;0;0;0;1;1;1;1;0;
Strain15;0;0;0;1;0;0;0;1;
Strain16;0;0;1;1;0;0;1;0;
Strain17;0;0;0;0;1;0;0;0
Strain18;0;0;1;1;0;0;1;0;
Strain19;0;0;0;1;0;0;0;1;

Strain1	0.4	0.4	0.066667	0.6	0.533333	0.466667	0.4	0.333333	0.4	0.333333	0.333333	0.2	0.466667	0.333333	0.6	0.266667	0.533333	0.333333
Strain2	0	0.333333	0.6	0.533333	0.466667	0.533333	0.333333	0.666667	0.6	0.6	0.466667	0.466667	0.6	0.6	0.533333	0.533333	0.6	
Strain3	0.333333	0.6	0.533333	0.466667	0.533333	0.333333	0.666667	0.6	0.6	0.466667	0.466667	0.6	0.6	0.533333	0.533333	0.6		
Strain4	0.533333	0.466667	0.4	0.466667	0.266667	0.333333	0.4	0.266667	0.133333	0.4	0.4	0.533333	0.333333	0.466667	0.4			
Strain5	0.2	0.266667	0.466667	0.266667	0.333333	0.4	0.4	0.533333	0.4	0.4	0.133333	0.466667	0.2	0.4				
Strain6	0.066667	0.266667	0.2	0.133333	0.2	0.2	0.333333	0.2	0.2	0.066667	0.266667	0.133333	0.2					
Strain7	0.333333	0.266667	0.2	0.266667	0.266667	0.266667	0.133333	0.266667	0.133333	0.333333	0.066667	0.266667						
Strain8	0.333333	0.266667	0.066667	0.2	0.333333	0.333333	0.066667	0.333333	0.133333	0.4	0.066667							
Strain9	0.333333	0.266667	0.266667	0.266667	0.266667	0.266667	0.266667	0.333333	0.333333	0.266667								
Strain10	0.2	0.066667	0.2	0.333333	0.2	0.2	0.266667	0.266667	0.2									
Strain11	0.133333	0.266667	0.266667	0	0.266667	0.066667	0.333333	0										
Strain12	0.133333	0.266667	0.133333	0.266667	0.2	0.333333	0.133333											
Strain13	0.266667	0.266667	0.4	0.333333	0.333333	0.266667												
Strain14	0.266667	0.266667	0.333333	0.2	0.266667													
Strain15	0.266667	0.066667	0.333333	0														
Strain16	0.333333	0.066667	0.266667															
Strain17	0.4	0.066667																

Appendix (4) The exact position of the retrieved 1500 bp amplicon that entirely covered a portion of the *16S rRNA* genetic sequences (acc no. CP012901.1). The green arrow refers to the starting point of this amplicon, while the cyan arrow refers to its end point.

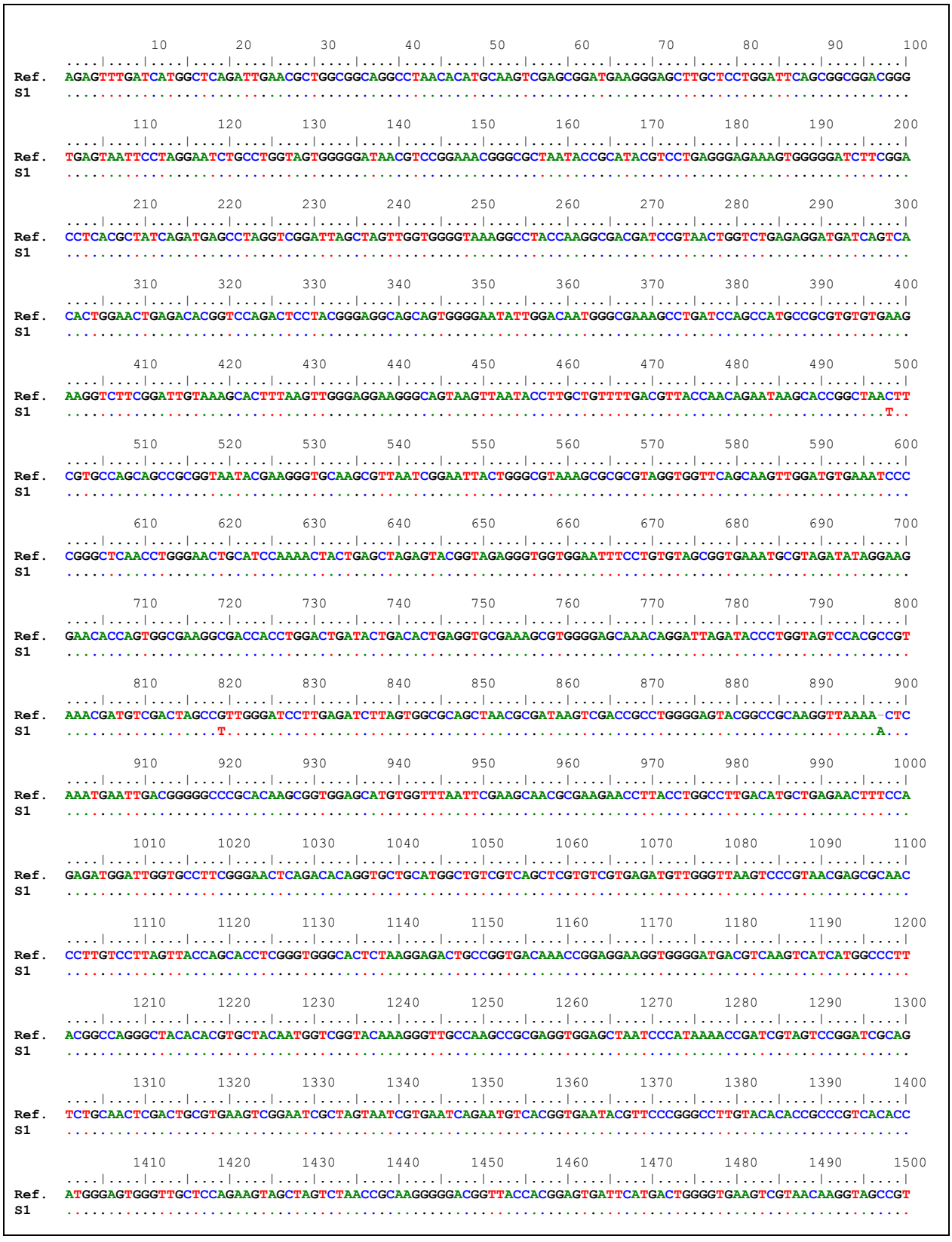


Appendix (5) The position and length of the 1500 bp PCR amplicons used to amplify a portion of the *16S rRNA* fragment. The amplified sequences were extended from 223007 to 224506 of the NCBI reference DNA sequence (GenBank acc. no. CP012901.1).

Amplicon	Referring locus sequences (5′ - 3′)	Length
16SrRNA DNA sequences	AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCG AGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATTCCTAG GAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATACCGCATACGT CCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCG GATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGA GAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGC AGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAA GAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATAC CTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCC GCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTA GGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAA AACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAAT GCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGAC ACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC GTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGC GATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACG GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTT ACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTC AGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC CCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAG GAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCC TTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCGC GAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGA CTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTT CCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAGTAG CTAGTCTAACCGCAAGGGGGACGGTTACCACGGAGTGATTCATGACTGGGGTGAAGT CGTAACAAGGTAGCCGTA*[1]	1500 bp

***[1] Notice; the reverse primer was placed in a reverse complement mode.**

Appendix (6) DNA sequences alignment of one local sample with its corresponding reference sequences of the 1500 bp amplicons of the *16S rRNA* DNA sequences. The symbol "ref" refers to the NCBI reference sequences, while symbols "S1" refer to the strain S1.



```
      .
Ref.  A
S1    .
```

Appendix (7) Accession number of tow Iraqi strains of *P. aeruginosa* in Gene bank were isolated from human sources in Baquba city/Diyala and each sequences has a symbol code (SL221814, SL8283).

GenBank ▾ Send to

Pseudomonas aeruginosa strain SL221814 16S ribosomal RNA gene, partial sequence

GenBank: MK503659.1

FASTA Graphics

Go to: ⊙

```
LOCUS       MK503659               1389 bp    DNA     linear   BCT 15-FEB-2019
DEFINITION  Pseudomonas aeruginosa strain SL221814 16S ribosomal RNA gene,
            partial sequence.
ACCESSION   MK503659
VERSION     MK503659.1
KEYWORDS    .
SOURCE      Pseudomonas aeruginosa
  ORGANISM  Pseudomonas aeruginosa
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1  (bases 1 to 1389)
  AUTHORS   Salman,L.A., Rasheed,H.R. and Farhan,A.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-FEB-2019) Biology department, Diyala University
            /College of Science, Diyala, Diyala, Iraq 32001, Iraq
COMMENT     Sequences were screened for chimeras by the submitter using
            geneious 10.

            ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
     source          1..1389
                     /organism="Pseudomonas aeruginosa"
```

Appendix

GenBank ▾ Send to: ▾

Pseudomonas aeruginosa strain SL8283 16S ribosomal RNA gene, partial sequence

GenBank: MK559695.1

FASTA Graphics

Go to:

```
LOCUS       MK559695               1391 bp    DNA     linear   BCT 01-MAR-2019
DEFINITION  Pseudomonas aeruginosa strain SL8283 16S ribosomal RNA gene,
            partial sequence.
ACCESSION   MK559695
VERSION     MK559695.1
KEYWORDS    .
SOURCE      Pseudomonas aeruginosa
  ORGANISM  Pseudomonas aeruginosa
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1  (bases 1 to 1391)
  AUTHORS   Al-Saadi,L.A., Al-Taai,H.R. and Al-Dulaimi,A.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (24-FEB-2019) Biology Department, Diyala University
            College of Science, Diyala, Diyala, Iraq 32001, Iraq
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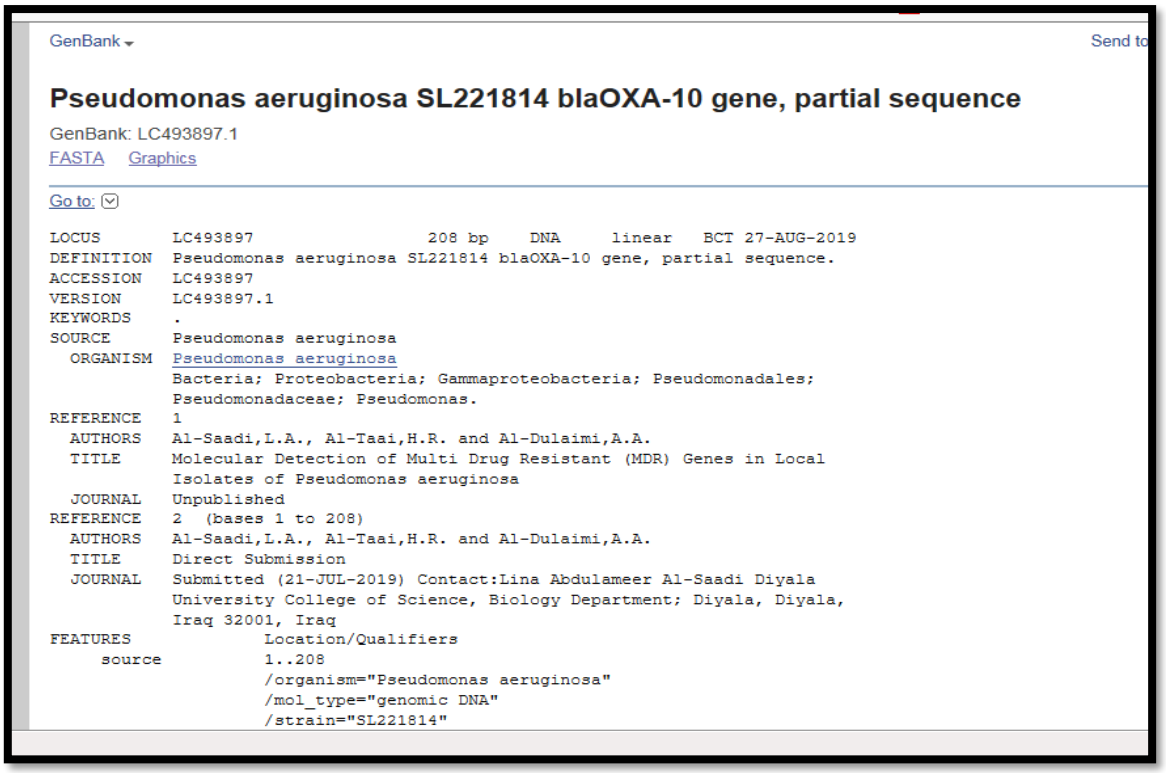

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Pseudomonas aeruginosa SL221814 blaOXA-10 gene, partial sequence

GenBank: LC493897.1

FASTA Graphics

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  TITLE     Molecular Detection of Multi Drug Resistant (MDR) Genes in Local
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  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 208)
  AUTHORS   Al-Saadi,L.A., Al-Taai,H.R. and Al-Dulaimi,A.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (21-JUL-2019) Contact:Lina Abdulameer Al-Saadi Diyala
            University College of Science, Biology Department; Diyala, Diyala,
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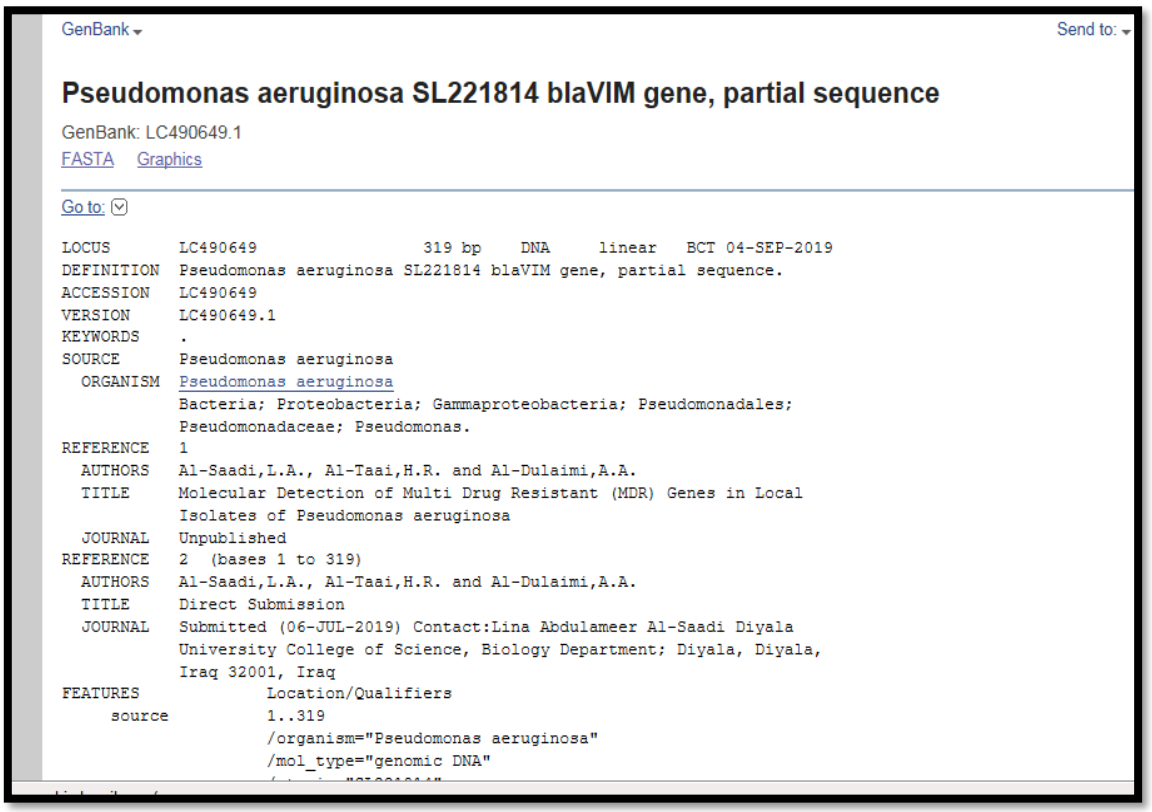

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Pseudomonas aeruginosa SL221814 blaVIM gene, partial sequence

GenBank: LC490649.1

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  TITLE     Molecular Detection of Multi Drug Resistant (MDR) Genes in Local
            Isolates of Pseudomonas aeruginosa
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 319)
  AUTHORS   Al-Saadi,L.A., Al-Taai,H.R. and Al-Dulaimi,A.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (06-JUL-2019) Contact:Lina Abdulameer Al-Saadi Diyala
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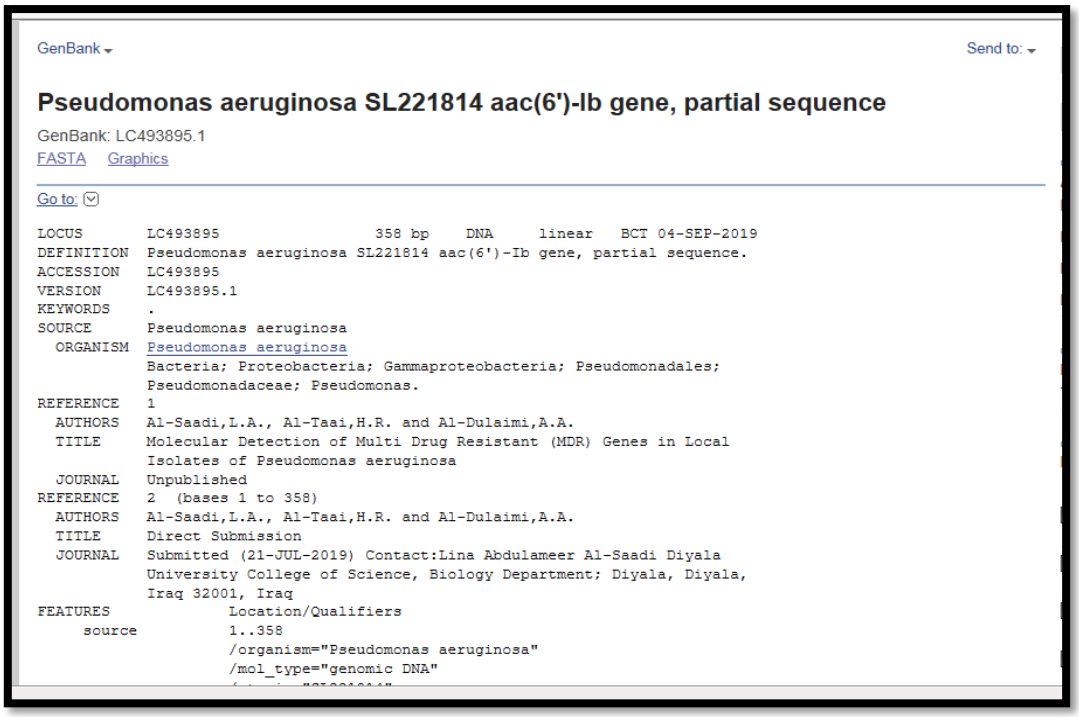

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Pseudomonas aeruginosa SL221814 aac(6')-Ib gene, partial sequence

GenBank: LC493895.1
FASTA Graphics

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  TITLE     Molecular Detection of Multi Drug Resistant (MDR) Genes in Local
            Isolates of Pseudomonas aeruginosa
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REFERENCE   2  (bases 1 to 358)
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  TITLE     Direct Submission
  JOURNAL   Submitted (21-JUL-2019) Contact:Lina Abdulameer Al-Saadi Diyala
            University College of Science, Biology Department; Diyala, Diyala,
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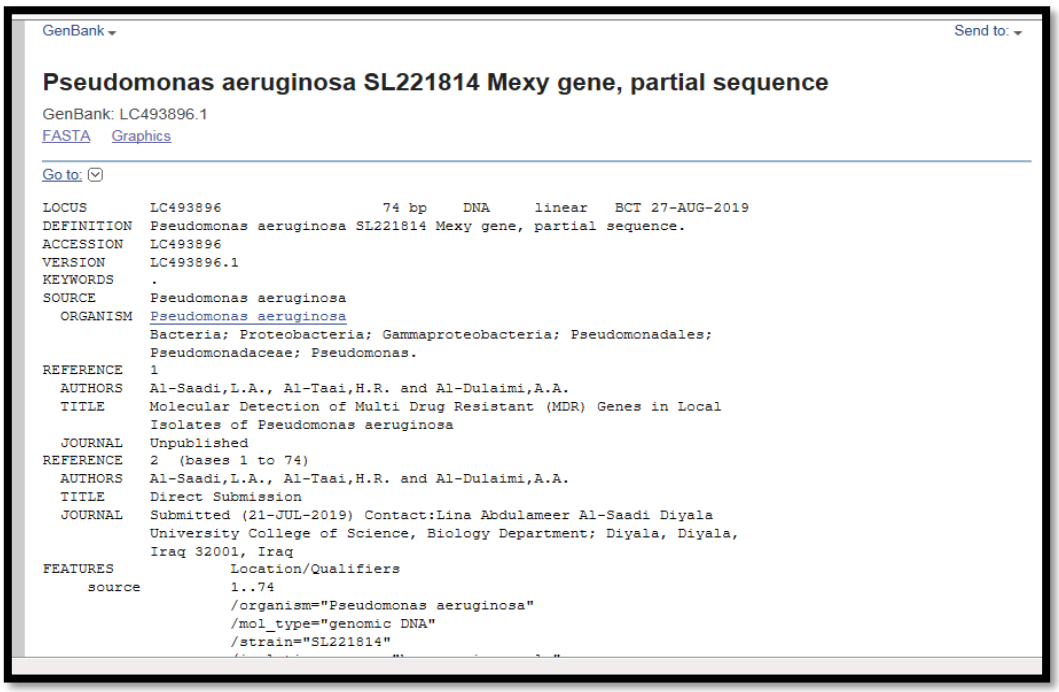

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Pseudomonas aeruginosa SL221814 Mexy gene, partial sequence

GenBank: LC493896.1
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            Isolates of Pseudomonas aeruginosa
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  TITLE     Direct Submission
  JOURNAL   Submitted (21-JUL-2019) Contact:Lina Abdulameer Al-Saadi Diyala
            University College of Science, Biology Department; Diyala, Diyala,
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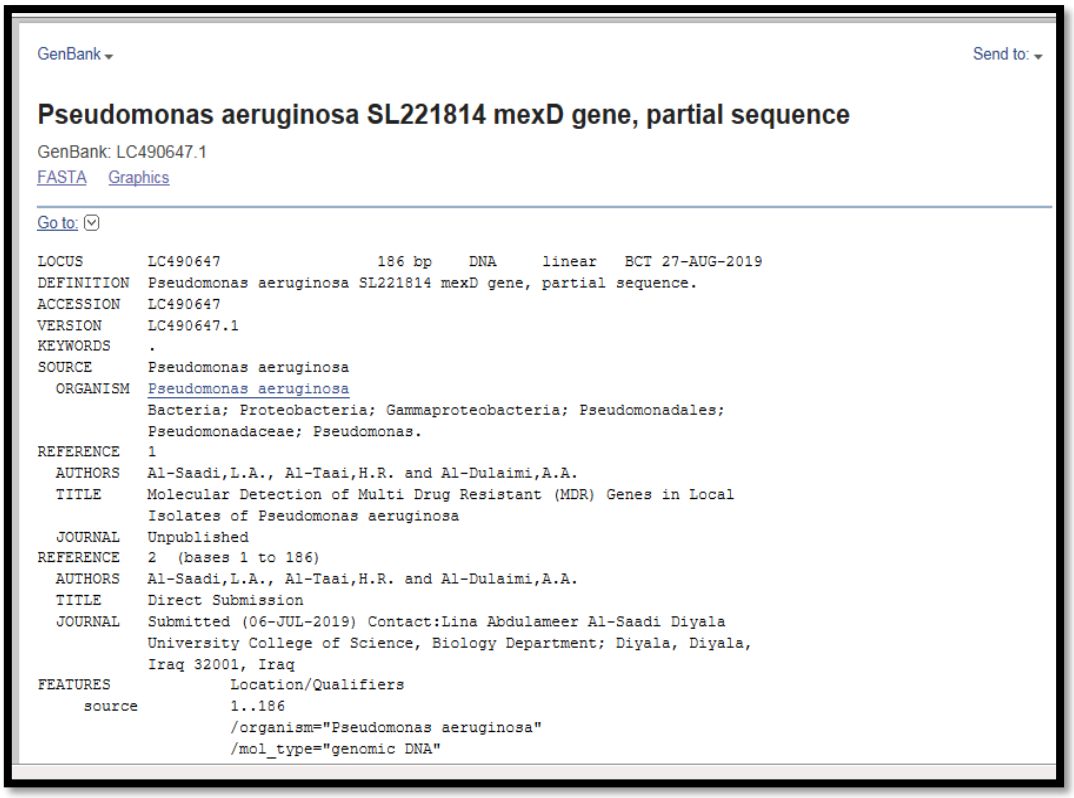

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Pseudomonas aeruginosa SL221814 mexD gene, partial sequence

GenBank: LC490647.1

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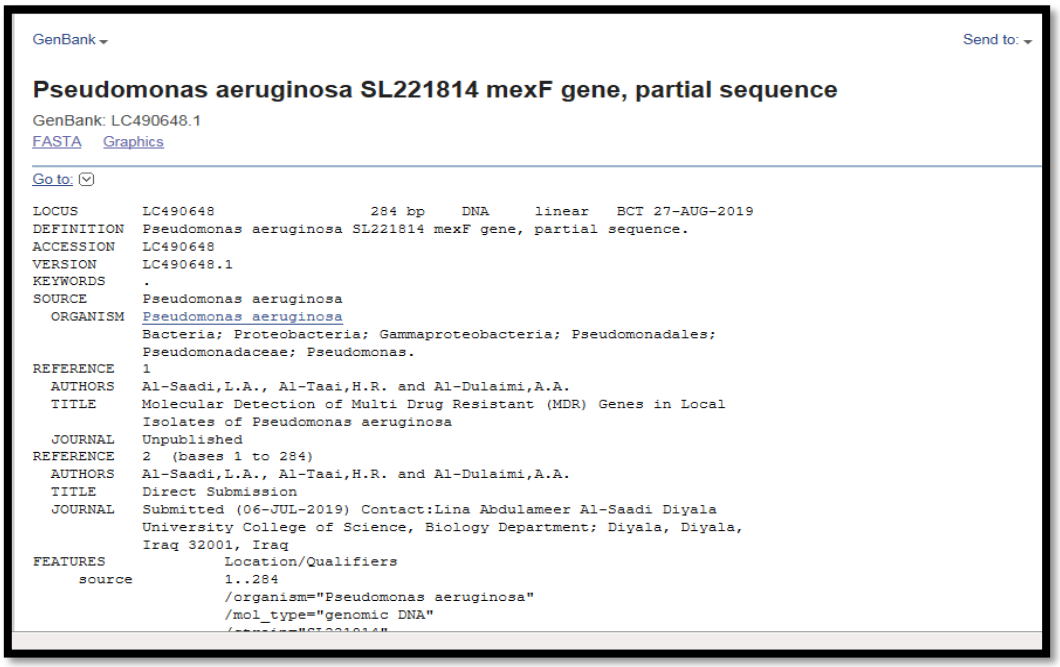

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Pseudomonas aeruginosa SL221814 mexF gene, partial sequence

GenBank: LC490648.1

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GenBank ▾ Send to: ▾

Pseudomonas aeruginosa SL8283 DNA, blaOXA10 gene, partial sequence

GenBank: LC495403.1

FASTA Graphics

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  AUTHORS   Salman,L.A., Al-Taai,H.R. and Farhan,A.A.
  TITLE     Molecular Detection of Multidrug Resistant(MDR) Genes in Local
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  JOURNAL   Unpublished
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  TITLE     Direct Submission
  JOURNAL   Submitted (02-AUG-2019) Contact:Lina Abdulameer Salman University
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Pseudomonas aeruginosa SL8283 DNA, blaVI gene, partial sequence

GenBank: LC495404.1

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  TITLE     Molecular Detection of Multidrug Resistant(MDR) Genes in Local
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GenBank ▾ Send to: ▾

Pseudomonas aeruginosa SL8283 aac(6')-Ib gene for aminoglycoside N-acetyltransferase, partial cds

GenBank: LC495320.1

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  AUTHORS   Salman,L.A., Al-Taai,H.R. and Farhan,A.A.
  TITLE     Molecular Detection of Multidrug Resistant(MDR) Genes in Local
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  JOURNAL   Unpublished
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  TITLE     Direct Submission
  JOURNAL   Submitted (01-AUG-2019) Contact:Lina Abdulameer Salman University
            of Diyala, College of Science, Biology Department; Baqubah, Diyala
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Pseudomonas aeruginosa SL8283 DNA, mexY gene, partial sequence

GenBank: LC495402.1

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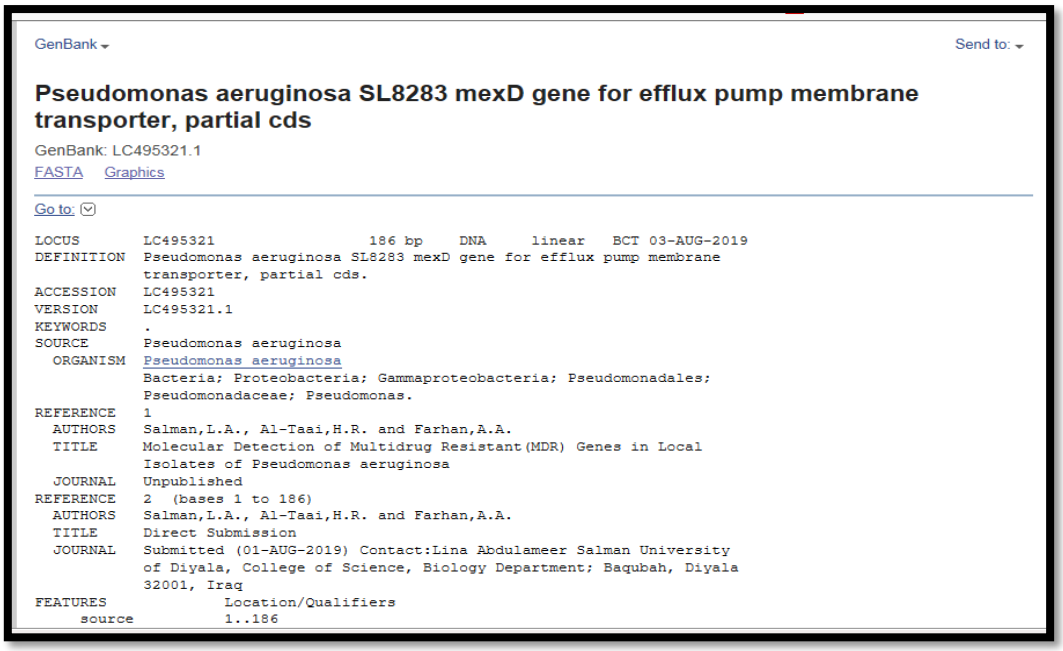

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Pseudomonas aeruginosa SL8283 mexD gene for efflux pump membrane transporter, partial cds

GenBank: LC495321.1

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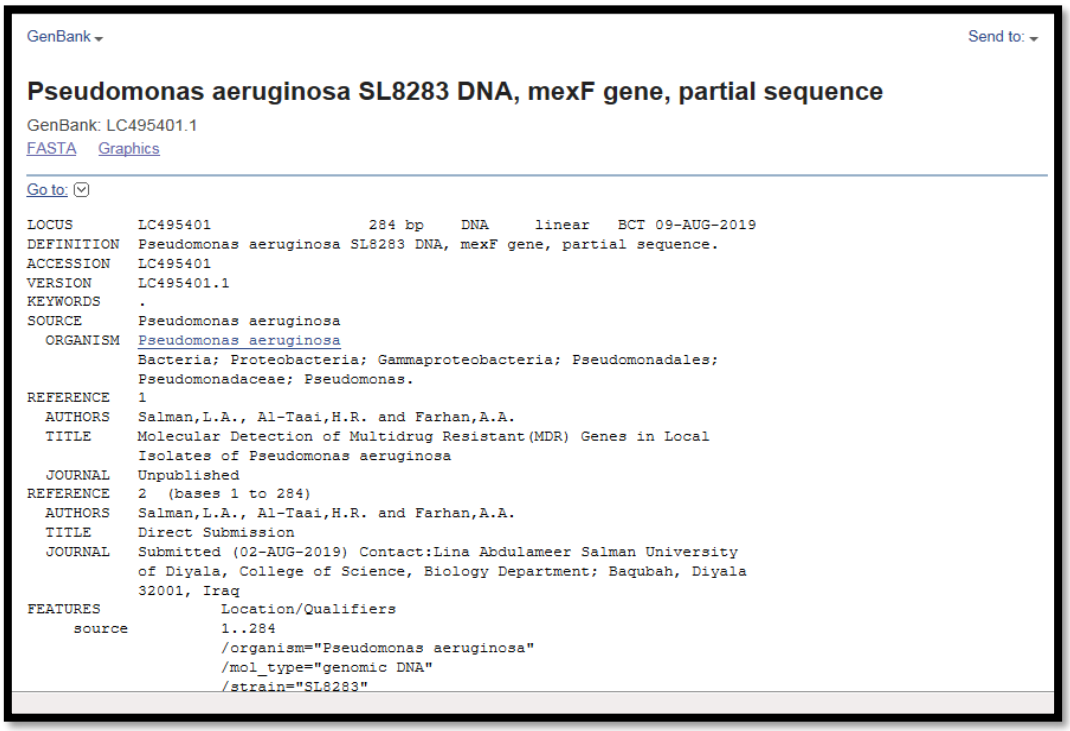

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Pseudomonas aeruginosa SL8283 DNA, mexF gene, partial sequence

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Appendix (8) In silico primer and probe design using IDT Primer Quest Tool

***bla*OXA-10**

>CP033439.1:6699855-6700655 *Pseudomonas aeruginosa* strain SP4528 chromosome, complete genome

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Parameter Set: RT-qPCR (Primers with Probe)
Sequence Name: OX10
Amplicon Length: 129

primer	Start	Stop	Length	Tm	GC%
Forward **GTCTTTCGAGTACGGCATTAG (Sense)**	35	56	21	60	47.6
Probe **ATTGACGGCTTCGGCAGAGAACTC (AntiSense)**	91	115	24	68	54
Reverse **CATTGGTAGCGCAGGATTTA (AntiSense)**	144	164	20	60	45

Appendix

***aac(6')Ib* gene**

>NC_009656, from 3860891 to 3861194 (304 bp); *Pseudomonas aeruginosa* PA7
CGGTACCTTGCCTCTCAAACCCCGCTTTCTCGTAGCATCGGATCGCTCGCAAGTTGCTCGGCGACGGGTCCG
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Parameter Set: RT-qPCR (Primers with Probe)
Sequence Name: aac
Amplicon Length: 100

primer	Start	Stop	Length	Tm	GC%
Forward **GTTTCTTCTTCCCACCATCC (Sense)**	204	224	20	60	50
Probe **AATGGAGAGCCGATTGGGTATGCC (AntiSense)**	254	278	24	68	54
Reverse **CCGTCACTCCATACATTGC (AntiSense)**	285	304	19	60	52.6

Appendix

MexY gene

>CP029093.1:3302185-3303375 *Pseudomonas aeruginosa* strain AR441 chromosome, complete genome

ATGCACATCCAATGGACCGGCTCGCTGCGCGGGCTGCTGGCGACCCTGGTCGCCCTATTCCTGCTGGGCT
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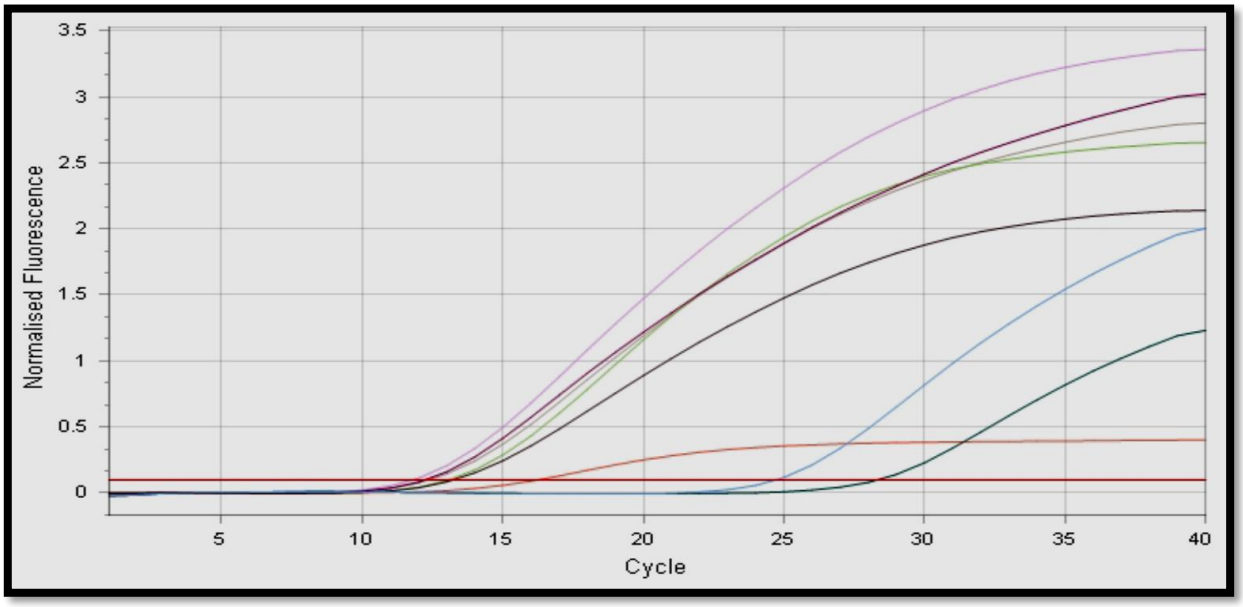
Parameter Set: RT-qPCR (Primers with Probe)

Sequence Name: MexY

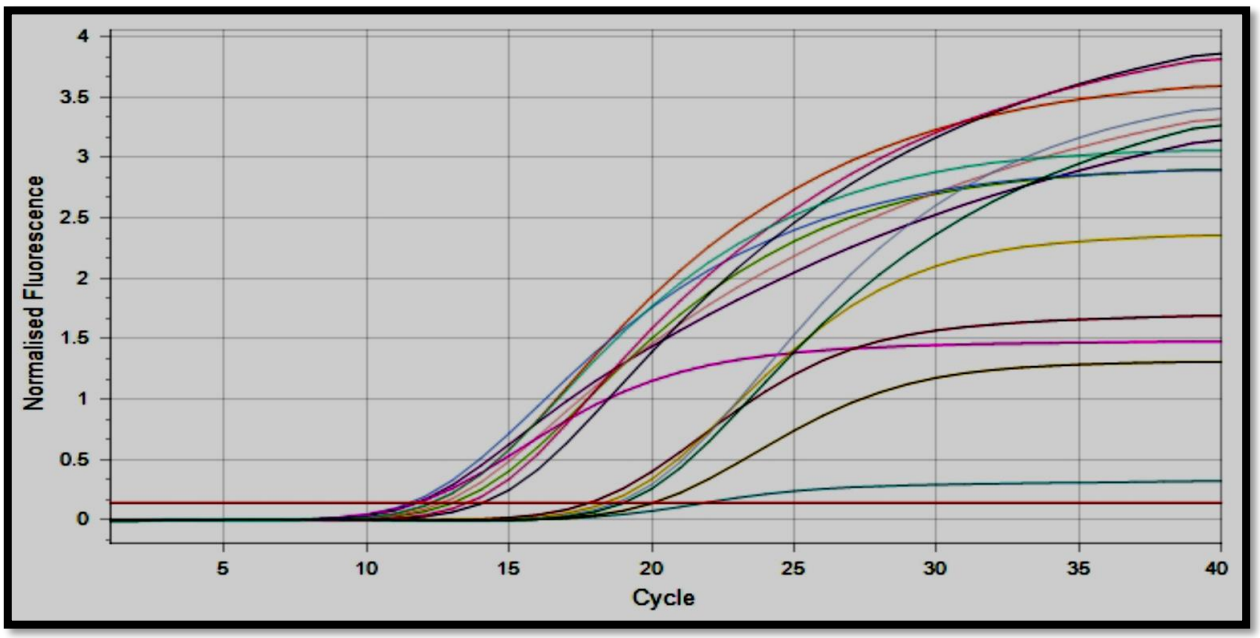
Amplicon Length: 112

Primer and probe	Start	Stop	Length	Tm	GC%
Forward TCGCCCTATTCCTGCTG (Sense)	35	56	21	60	47.6
Probe AGGAGGGCCAGGATGTTC (AntiSense)	91	115	24	64	54
Reverse AGTTCGCTGGTGATGCC (AntiSense)	144	164	20	60	45

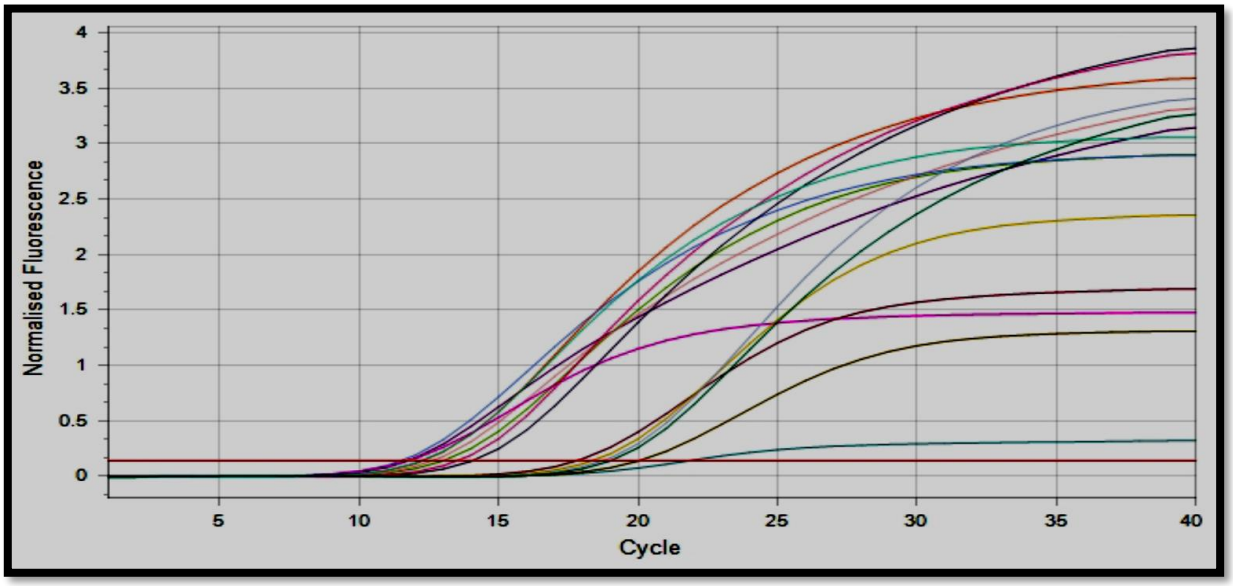
Appendix (9) qPCR Amplification plots of *bla*$_{OXA-10}$ expression for *P. aeruginosa* isolates .



Appendix (10) qPCR Amplification plots of *aac(6')Ib* expression for *P. aeruginosa* isolates .



Appendix (11) qPCR Amplification plots of *MexY* expression for *P. aeruginosa* isolates .



جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة ديالى
كلية التربية للعلوم الصرفة
قسم علوم الحياة

الكشف الجزيئي عن بعض جينات المقاومة المتعددة وتأثير اوكسيد الزنك النانوي كبديل عن المضادات الحيوية لبكتريا الزوائف الزنجارية

أطروحة مقدمة
إلى مجلس كلية التربية للعلوم الصرفة بجامعة ديالى كجزء من متطلبات نيل درجة دكتوراه فلسفة في علوم الحياة

من قبل

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بإشراف

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الخلاصــة

تم جمع (326) عينة سريرية من إصابات الحروق والجروح والبلغم والبول والتهاب الأذن الوسطى من مرضى مستشفيات مختلفة في محافظة ديالى خلال الفترة من شباط إلى اب 2018. أظهرت النتائج أن العزلات البكتيرية ظهرت في 293 من هذه العينات. أظهر الكشف باستخدام الطرق الميكروبيولوجية التقليدية ونظام التشخيص VITEK 2 والكشف الجزيئي باستخدام جين *16S rRNA* ان81(27.6٪) من العزلات كانت لبكتريا *Pseudomonas aeruginosa*.

تم التحقق من مقاومة و حساسية عزلات بكتريا *P. aeruginosa* ضد (18) نوع من المضادات الحيوية من مجاميع مختلفة بوساطة طريقة انتشار الاقراص القياسية (كيربي باور) لتقييم مقاومتها. كانت هناك زيادة كبيرة ($p < 0.01$) في معدل مقاومة بكتريا *P. aeruginosa* للمضادات الحيوية المختلفة, اذ بلغت 93.82٪ لمضاد Amoxicillin-Clavulanic acid و 90.12٪ لـ Streptomycin و 87.65٪ لمضاد Ceftriaxone و 85.18٪ لمضاد Ticarcillin و 85.18٪ لمضاد Cefotaxime و 85,18% لمضاد Gentamicin و80,24% لمضاد Cefepime و 75,30% لمضاد Ceftazidime و 74,07% لمضاد Piperacillin و 72,83% لمضاد Levofloxacin و 71.60٪ لمضاد Ticarcillin / Clavulanic بالمقارنة مع معدل مقاومتها لكل من المضادات oflaxacin و ciprofloxacin و Tobramicin و Amikacin و Aztreonam اذ كانت (69.13 ٪) و (67.90 ٪) و (65.43 ٪) و (56.79 ٪) و (50.61 ٪) على التوالي ، في حين كان هناك انخفاض في معدل مقاومة البكتريا لمضاد Meropenem (23.45 ٪) و Imepenem (11.11٪).

أظهر اختبار حساسية المضادات الحيوية لعزلات بكتريا *P. aeruginosa* أن 20 عزلة (24.69٪) كانت حساسة لمضادات متعددة (MDS) و 25 عزلة (30.8٪) متعددة المقاومة للمضادات الحيوية MDR و27 عزلة (33.33٪) شديدة المقاومة للمضادات الحيوية (XDR) و 9 عزلات (11,11%) مقاومة للمضادات الحيوية كافة (PDR). استنادًا إلى نتائج اختبار الحساسية، وجد ان 19(23.45٪) من عزلات *P. aeruginosa* كانت مقاومة لواحد على الأقل من مضادات الكاربابنيم Carbapenems. ظهرت مقاومة البكتريا لمضادات Carbapenems من خلال اختبار انتشار الاقراص ان 9عزلات (11.11٪) مقاومة لكل من مضادي Meropenem و Imipenem وفي 10عزلات (12.34٪) مقاومة لمضاد Meropenem وحده. في الدراسة الحالية تم اختيار (19) عزلة من بكتريا الزوائف الزنجارية *P. aeruginosa* المقاومة لمضادات Carbapenems للتحري عن الجينات المشفرة لانزيمات البيتالاكتاميز β-lactamase والجينات المقاومة للأمينوكلايكوسيدات و جينات مضخات الدفق باستخدام تقنية PCR.

استخدمت طريقة التخفيف المضاعف لتحديد التركيز المثبط الأدنى (MIC) و التركيز القاتل الأدنى (MBC) لثلاثة من المضادات الحيوية، وأظهرت النتائج أن هناك اختلافات في قيم MIC و MBC. استطاعت جميع العزلات 19(100٪) ان تنمو في تراكيز عالية من مضاد Gentamicin (تراوحت قيم MIC من 64-1024 ميكروغرام/مل) و (تراوحت MBC من 256- > 1024 ميكروغرام/مل) و تراوحت MIC لمضاد Ceftazidime بين (64- 512 ميكروغرام/مل) وكانت قيمة MBC (128- 1024 >). كان مضاد Imipenem أكثر المضادات الحيوية فعالية اذ تراوحت قيم MIC من (2–256 ميكروغرام/مل) وتراوحت MBC من (4- 512 ميكروغرام/مل). تراوحت قيمة MIC لجسيمات الزنك النانوية ZnO بين (325-5200 ميكروغرام/مل) في عزلات *P. aeruginosa* المقاومة للـ Carbapenem.

تم التحري عن جينات البيتالاكتاميز واسعة الطيف ESBLs (bla_{OXA-10}) و (bla_{PER}) بوساطة تقنية PCR. اظهرت نتائج الترحيل الكهربائي في هلام الاكاروز لمنتج PCR باستخدام بادئات محددة لهذه الجينات أن 12(63.15٪) من العزلات كانت موجبة لجين bla_{OXA-10}. بينما لم تحمل أيا من عزلات بكتريا *P.aeruginosa* المقاومة لمضادات Carbapenem جين ESBL bla_{PER}. وجد ان من بين 19 عزلة من عزلات بكتريا *P. aeruginosa* المقاومة للكاربابينيم Carbapenem كانت 16(84.21٪) فقط منتجة MβL. و ان من بين (16) عزلة من العزلات التي اعطت نتيجة موجبة لانزيمات البيتالاكتاميز المعدنية (Metallo Beta-lactamase) بالكشف المظهري ، حسب نتائج PCR كانت 9 عزلات (56.25٪) فقط تحمل جينات bla_{VIM}، في حين أن 4 عزلات (25٪) كانت تحمل جينات bla_{NDM} ، ولم يتم الكشف عن وجود جين bla_{IMP} بين عزلات البكتريا المقاومة للـ Carbapenem في هذه الدراسة.

في الدراسة الحالية، تم الكشف عن ثلاثة (3) من الجينات المحورة للامينوكلايكوسيدات (AME) وهي الجينات *aac (6 ')-Ib* ، *aac(3')-II* و *ant (4 ')–IIb*. أظهرت النتائج باستخدام طريقة Uniplex PCR أن *aac (6 ') - Ib* كان من ضمن جينات AME الأكثر انتشارًا اذ وجد في 19/18(94.73٪) من العزلات تلته جينات ant (4') - IIb و aac (3 ')-II التي كانت معدلاتها 10.52٪ (19/19) و 5.26٪ (19/1) على التوالي.

استنادًا إلى نتائج Uniplex PCR، وجد أن 19 عزلة من عزلات بكتريا *P.aeruginosa* المقاومة للكاربابنيم Carbapenem أعطت نتائج إيجابية لجين MexY لنظام الدفق، واستناداً إلى نتائج Multiplex PCR، فان 18 (94.7٪) من العزلات كانت تحمل جينات MexB و MexF، في حين أن 17 عزلة (89.47 ٪) عزلة كانت تحمل جين MexD.مما قد يدل على انتشار هذا النوع من المقاومة في

العزلات البكتيرية الحالية. وكشفت النتائج أن 19/18 (94.7 ٪) من العزلات كانت تحمل كل من جينات AMEs وجينات الدفق. ويلاحظ أن معظم العزلات كانت تحمل أكثر من نوع واحد من جينات مضخة التدفق بالاشتراك مع واحد أو أكثر من جينات AME. و تبين أيضًا أن 19/12(63.15٪) من العزلات كانت تحمل كلاً من جينات ESβL وجينات الدفق.

بناءً على التحري عن جين Integrase للتحري عن انتكرون الصنف الاول integron، أظهر اختبار PCR أن 19 عزلة(100٪) من عزلات بكتريا *P. aeruginosa* المقاومة لـ Carbapenem كانت إيجابية لجين Integrase، مما يؤكد الانتشار العالي للغاية للانتكرون الصنف الاول class (1) integron في مستشفيات بعقوبة في محافظة ديالى. أظهر تحليل المناطق المتغيرة من الانتكرون الصنف الاول integron وجود اربعة (4) أحجام لاجزاء مختلفة بلغت 300 ، 400 ، 600 و 1500 زوج قاعدي. وبينت النتائج الحالية أيضًا أن 9 عزلات (47.36٪) تحمل انتكرون الصنف الاول مع أحجام تبلغ حوالي 1.5 كيلو بايت، كذلك ظهرت 2(10.52٪) عزلة بأحجام تبلغ حوالي 600 زوج قاعدي و 400 زوج قاعدي وكذلك وجد ان 3 عزلات (15.78٪) كانت بأحجام بلغت تقريبًا 300 زوجا قاعديا. غير ان كشف تحليل المنطقة المتغيرة في integron أن 19/3(15.78٪) عزلة كانت تفتقر إلى أشرطة الجينات، مما يشير إلى انخفاض ظهور انتكرونات الصف الاول الفارغة بين هذه السلالات.

كشفت نتائج التنميط الجيني بتقنيةERIC-PCR لـ 19عزلة من بكتريا *P.aeruginosa* المقاومة لـ Carbapenem أن 19(100٪) منها اظهرت حزمة تضخيم واحدة على الأقل. واظهر التنميط بطريقة ERIC-PCR مجموعتين A وB مع 6 عزلات فريدة من نوعها، والتي تبين أنماط مقاومة للمضادات الحيوية مماثلة داخل المجموعة نفسها.

تم عزل سلالتين من سلالات بكتريا *P. aeruginosa* المقاومة للكاربابنيم Carbapenem ذات المصادر البشرية في مدينة بعقوبة / ديالى وكان لكل تسلسل رمز شفرة وهي (SL221814، SL8283) 16 S rRNA قدمت إلى بنك الجينات العام. تم نشر هذه السلالة في المركز الوطني لمعلومات التكنولوجيا الحيوية (NCBI)، في حين تم تسجيل قاعدة بيانات السلالة في بنك بيانات الحمض النووي في اليابان (DDBJ) وبنك الجينات لتسلسل الحمض النووي مع رقم تسلسلي لستة(6) جينات مقاومة للمضادات. تم قبول كل هذه التسلسلات في بنك الجينات واتخذ كل تسلسل رقم الانضمام (MK503659 و MK559695). نجحت الدراسة الحالية في الاعتماد على كلتا العزلتين (MK503659) Pa7 و (Pa10 MK559695) لتكون مؤشرات قياسية كنوع من السيطرة الإيجابية مثل العزلات المرجعية لمقارنة نتائج

التعبير الجيني لـجينات 16 S rRNA، bla_{OXA10} ، bla_{VIM}، aac (6) Ib ، MexY ، MexD و MexF لعزلات أخرى بناءً على هذين العزلتين.

تم تصميم اختبار التفاعل اللحظي Taq Man qRT-PCR للكشف عن خصائص الجينات وتوصيفها،.اذ تم إجراء تضخيم جزء من الرنا الرسول (mRNA) مع تفاعل التضخيم الرئيسي التالي باستخدام برنامج RT-PCR أحادي الخطوة: تم تصميم أجهزة المجس prob والبادئات الخاصة primers بأنواع الجينات bla_{OXA10} و *aac (6 ') Ib* و MexY كهدف لتحديد تعبير خمسة عزلات (5) من (19) من عزلات *P. aeruginosa*. وتم توزيع المصادر المختلفة لهذه العزلات على النحو التالي: (1 جروح ، 1حروق ،1 بول ، 1 بلغم و 1 أذن) بالإضافة إلى الجين المرجعي *rps*L ذو الحجم (201 زوج قاعدي) لتقييم التعبير الجيني لجينات المقاومة هذه.

أظهر تعبير جين bla_{OXA10} أن أعلى مستوى من folding بمعدل يبلغ 4.06 وجد في حالة العزلات المعاملة بمضاد Ceftazidime عند تركيز 128 ميكروغرام/مل. وأظهرت ثلاث عزلات هي (Pa5 و Pa8 و Pa10) تعبيرًا مفرطًا عن جين bla_{OXA10} مع معدلات عالية من قيم MIC من مضاد Ceftazidime يبلغ (256-512) ميكروغرام/مل. وأظهرت قيم sub MIC لجزيئات ZnO النانوية تأثيرا فعالاً في تعبير كل من جينات bla_{OXA10} و *rps*L (الجينات المستهدفة) اذ احدثت فرقا معنويا كبيرا وفقا لقيم مربع كاي $\chi 2$ عند مستوى معنوية $P < 0.05$ من بين العزلات المستهدفة. أدت المعاملة باستخدام جزيئات ZnO النانوية إلى انخفاض في قيمة التعبير الجيني في أربعة عزلات لجين bla_{OXA10} والتي كانت تقريبًا بمعدل (0.45) بتركيز يبلغ 325 مايكروغم/مل والتغيير من 0.001 إلى 0.13 ضعفًا في التعبير الجيني bla_{OXA10} كان بسبب للتعرض الفعال لـ 325 مايكروغم/مل من جزيات ZnO النانوية التي أثرت بشدة على تعبيرجين bla_{OXA10}. من ناحية أخرى، لم تتأثر العزلة (Pa10) بـ subMIC لجزيئات ZnO النانوية اذ كانت قيمة التغيرfold 1.04. كذلك لم يكن هناك أي تغيير و لا تناقص ولا زيادة في التعبير(fold) اذ بقيت عند المدى (1).

بين تعبير جين *aac (6 ') Ib* أن أعلى مستوى من التغيرfold كان بمعدل 14.77 في حالة العزلات التي تم معاملتها بمضاد Gentamicin بتركيز256 مايكروغم/مل. أظهرت العزلتان (Pa5 و Pa10) تعبيرًا مفرطًا من الجين *aac (6 ') Ib* مع وجود تغيرات في التعبير foldig 30.22 و 32.08 على التوالي. أدت المعاملة باستخدام جزيئات ZnO النانوية إلى انخفاض في قيمة التعبير الجيني في (3) عزلات لجين *aac (6 ') Ib* وهي 0.44،0.001 و 0.63 مايكروغم/مل. من ناحية أخرى، لم تتأثر العزلتان (Pa5 و Pa7) باستخدام subMIC اذ كانت قيم التغير في التعبير (folding) 2.56 و 11.36. اذ لم يكن هناك

أي زيادة في التعبير، ولم يتم تأثرها بواسطة جزيئات ZnO NPs النانوية. لذلك ، يمكن أن تكون هذه الدراسة أول دراسة لتقدير التعبير الجيني لجين *aac (6')Ib* لبكتريا *Pseudomonas aeruginosa*.

اوضح تعبيرجين الدفق MexY أن أعلى مستوى من التغير بالتعبير Fold بمعدل 8.16 وجد في حالة العزلات التي تم معاملتها بمضاد Gentamicin بتركيز 256 ميكروجرام/مل. أظهرت عزلة واحدة (Pa2) تعبيرًا مفرطًا عن الجين MexY اذ بلغ حوالي 25.52. أدت المعاملة باستخدام جزيئات ZnO NPsالنانوية إلى انخفاض في قيمة التعبير الجيني في جميع العزلات الخمسة لجين MexY بمعدل قدره 0.39 عند التركيز 325 ميكروغرام/مل. ويرجع ذلك إلى التأثير الفعال لـتركيز 325 مايكروغم/مل من جزيئات ZnO NPs والتي أثرت بشدة على تعبيرجين MexY.

Introduction

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Literature Review

Chapter Two

Materials and Methods

Chapter Three

Results and Discussion

Conclusions and Recommendations

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Appendix