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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
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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*

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

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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.