Molecular detection of OprD and ExoA in Pseudomonas aeruginosa and antibiotics resistance

Cite as: AIP Conference Proceedings **2475**, 030006 (2023); https://doi.org/10.1063/5.0103074
Published Online: 31 March 2023

Ruqaiya Mahmoud Saleh and Anfal Shakir Motib

Molecular Detection of OprD and ExoA in Pseudomonas Aeruginosa and Antibiotics Resistance

Ruqaiya Mahmoud Saleh[a)] and Anfal Shakir Motib[b)]

Department Of Microbiology, College Of Medicine, University Of Diyala, Diyala,, Iraq.

[a)] *Corresponding Author: ruqaia.mahmood@uodiyala.edu.iq*
[b)] *anfal_shaker@yahoo.com*

Abstract. The current study include the diagnosis of 12 isolates of Pseudomonas aeruginosa from a different clinical specimens wounds, burns, urine and vagina of inpatients and patients attended at Baqubah Teaching Hospital and Al-Batoul Teaching Hospital/Diyala Province. All isolates were diagnosed based on microscopic and biochemical tests. In this study, polymerase chain reaction assay was used for the molecular detection of P. aeruginosa that was obtained from wounds, burns, and urine and vagina samples. Two genes were targeted as specific markers for P. aeruginosa namely the outer membrane lipoprotein oprD gene which coding for Carbanems resistance, mainly for Imipenem and exoA gene which coding for protein synthesis inhibition and 12 isolates of P. aeruginosa were employed in this study and boiled bacterial suspension was used as a DNA template. The results showed that 10 isolates of P. aeruginosa gave positive PCR product for oprD gene with amplified size 193 bp and exoA gene with amplified size 397 bp and 2 isolates gave negative PCR product for both genes. This research indicates that molecular detection of P. aeruginosa that was isolated from wounds, burns, urine, and vagina samples employing oprD and exoA genes using PCR technique is a rapid method for bacterial diagnosis. In addition, antibiotic sensitivity was conducted P. aeruginosa isolates 1 and 9 which showed resistance to all antibiotics. Interestingly, these isolates did not appear PCR product for exoA or oprD, which indicated that these two genes play a role in antibiotic resistance in P. aeruginosa. The current study include the diagnosis of 12 isolates of *Pseudomonas aeruginosa* from a different clinical specimens wounds, burns, urine and vagina of inpatients and patients attended at Baqubah Teaching Hospital and Al-Batoul Teaching Hospital/Diyala Province. All isolates were diagnosed based on microscopic and biochemical tests. In this study, polymerase chain reaction assay was used for the molecular detection of *P. aeruginosa* that was obtained from wounds, burns, and urine and vagina samples. Two genes were targeted as specific markers for *P. aeruginosa* namely the outer membrane lipoprotein *opr*D gene which coding for Carbanems resistance, mainly for Imipenem and *exo*A gene which coding for protein synthesis inhibition and 12 isolates of *P. aeruginosa* were employed in this study and boiled bacterial suspension was used as a DNA template. The results showed that 10 isolates of *P. aeruginosa* gave positive PCR product for *opr*D gene with amplified size 193 bp and *exo*A gene with amplified size 397 bp and 2 isolates gave negative PCR product for both genes. This research indicates that molecular detection of *P. aeruginosa* that was isolated from wounds, burns, urine, and vagina samples employing *opr*D and *exo*A genes using PCR technique is a rapid method for bacterial diagnosis. In addition, antibiotic sensitivity was conducted *P. aeruginosa* isolates 1 and 9 which showed resistance to all antibiotics. Interestingly, these isolates did not appear PCR product for *exoA* or *oprD*, which indicated that these two genes play a role in antibiotic resistance in *P. aeruginosa.*

Keywords. Pseudomonas aeruginosa, Antibiotics resistance, PCR detection, oprD, exoA.

INTRODUCTION

Pseudomonas aeruginosa is gram-negative bacteria with bacilli shape and motile existed nearly everywhere in the environment such as soil, water, plants, animals and humans [1]. *P. aeruginosa* is an aerobic bacillus can be considered an opportunistic pathogen. It is a highly flexible microorganism capable of tolerating conditions of low oxygen, with low nutrient levels, it can live and grow at temperatures ranging from 4-42°C [2]. It is especially

1st International & 4th Local Conference For Pure Science (ICPS-2021)
AIP Conf. Proc. 2475, 030006-1–030006-7; https://doi.org/10.1063/5.0103074
Published by AIP Publishing. 978-0-7354-4327-3/$30.00

dangerous for people who have serious wounds, cystic fibrosis, or cancer. *P. aeruginosa's* infection strategy relies on the production of many cell-associated and secreted molecules, such as different types of enzymes and toxins [3].

Furthermore, *P. aeruginosa's* capacity to form biofilm is thought to be a key factor in chronic infections. Biofilms are a complex of microbial cells enclosed in an extracellular matrix made up of proteins, extracellular DNA, and exopolysaccharides that provide bacteria with a safe lifestyle and are extremely challenging and costly to treat by antimicrobial compounds [4]. Its wide metabolic flexibility allows it to thrive not only in a variety of aquatic and terrestrial environments, but also in the human host*, P. aeruginosa* infections can become established at various host sites and progress into life threatening acute or chronic infections [3,5].

*Opr*D gene is a Carbapenem-specific porin which reduction or absence causes primary Carbapenem resistance. Porins are three-partite protein channels in *P. aeruginosa's* outer membrane that selectively transfer hydrophilic molecules based on their size and charge [6]. Exotoxin A is a toxin that inhibits protein synthesis by causing ADP ribosylation of eukaryotic elongation factor, which is similar to the mechanism of feat of diphtheria poison. Purified exotoxin A is very fatal to animals, humans and primates. In hospitalized patients, exotoxin A is a significant virulence factor of *P. aeruginosa* in burn ward, ICUs, CCUs, and ITUs [7]. In this paper, PCR procedure had been used for rapid and specific detection of *P. aeruginosa* that was isolated from wounds, burns, urine and vagina samples by amplifying *opr*D and *exo*A genes and determine antibiotics sensitivity.

MATERIALS AND METHODS

Bacterial Isolates

Clinical isolates of *P. aeruginosa* were 12 isolates, which have been isolated from a different clinical specimens (wounds, burns, urine and vagina). All isolates were diagnosed based on bacteriological and biochemical tests. These isolates gave a positive results for oxidase, catalase and citrate utilization tests.

Antibiotics Susceptibility Testing

The sensitivity test procedure was done according to CLSI [8], as the following steps: -

1. Mueller-Hinton agar plates were used for the use of rapidly growing species in the Kirby-Bauer method. The solvent was sterile in the plates and had a depth of around 4 mm.
2. Pure culture has been used as inoculum 2-4 related colonies have been selected and transferred to around 5ml of standard sterile saline. To get an average number equal to (1.5x108) CFU/ml, the turbidity of microbial suspension was compared with the turbidity of the McFarland Standard (0.5).
3. A cotton swab from the microbial suspension was used to gently and evenly inoculate the surface of the plates which were read after 24 hr by measuring the zone of inhibition.

Molecular Methods

DNA Extraction

It was extracted the genomic DNA from bacterial growth according to the [9]. Primers selection (Forward and Reverse) used in PCR were specific for *opr*D and *exo*A genes according to the manufacture company and it was prepared in current study. The sequence of primer used in this study is given in table (1). Gel electrophoresis was used to analyze the results [10].

TABLE 1. The primers sequences which were used for amplifying *opr*D and *exo*A.

Genes		sequence (5'-3')	Products bp
oprD	F	5`-GCGCATCTCCAAGACCATG-3`	193
	R	5`-GCCACGCGATTTGACGGAG-3`	
exoA	F	5`-GACAACGCCCTCAGCATCACCAGC-3`	397
	R	5`-CGCTGGCCCATTCGCTCCAGCGCT-3`	

PCR Test

PCR techniques can generate millions of copies of a single segment of DNA sequence that may be a gene or clusters of genes with high fidelity in 3 to 4 [11]. Multiplex PCR is a technique that uses a chain of primers to amplify multiple target series in a single reaction, while standard PCR uses only one pair of primers to amplify a single sequence [12]. Boiled bacterial suspension was used as a DNA template. Two different primers were used, the first was the oprD primer for amplifying *opr*D gene [13].

F:5`- GCGCATCTCCAAGACCATG-3`, R:5`-GCCACGCGATTTGACGGAG-3`, with amplified size 193 bp and the second was exoA primer for amplifying *exo*A gene F:5`-GACAACGCCCTCAGCATCACCAGC3`, R:5`CGCTGGCCCATTCGCTCC AGCGCT-3`with amplified size 397bp. The PCR with 2µl DNA was performed in a final volume of 20µl with 10µl 2x Go Taq Green Master Mix, 1µl for each primer and 6µl dH2O. For *exo*A gene, the PCRs conditions started with an initial denaturation step at 95ºC for 5 min followed by 40 cycles each of 95ºC for 30 sec, 68ºC for 30 sec and 72ºC for 1min followed by a 7min extension period at 72ºC and soaking at 10ºC for unlimited time. With the *opr*D gene, the conditions were the same as above except that the annealing temperature was 55 ºC. 5µl of the PCR product were electrophoresed on a 1.5% agarose gel, supplied with ethidium bromide with final concentration 10µg/µl, visualized under UV light and photographed.

RESULTS AND DISCUSSION

Detection of OprD and ExoA Genes by PCR

Traditional microbiological methods for identifying *P. aeruginosa* from environmental samples are accurate, but they take a long time to complete. By amplification of gene sequences specific to a particular organism, PCR has the ability to quickly distinguish microbial species [14]. PCR uses primers that are 20-30 nucleotide sequences homologous to the ends of the genomic DNA region to be amplified to detect bacteria. The conventional PCR procedure consists in a thermocyclic process of repetitive cycles of DNA denaturation, annealing and extension through a thermo stable DNA polymerase. The amplified sequence is detected using agarose gel electrophoresis with fragment length as an identification indicator [15].

The amplify genes of *P. aeruginosa* isolates are shown in figure 1 and 2 all isolates of *P. aeruginosa* were positive for *opr*D gene with amplified size 193 bp as shown in figure (1) also gave positive for *exo*A gene with amplified size 397 bp as shown in figure (2) at a percentage of 83.3% except isolates 1 and 9 were negative for *opr*D and *exo*A genes at a percentage 16.7%. When comparing the results of our study with other studies found that Wolska and Szweda [16], showed that the PCR detected *exo*A gene in 55 isolates 88.7% out of 62 isolates of *P. aeruginosa* were originally isolated from a variety of clinical specimens, this result agree with the present study. The percentage of *exo*A gene in current study was closed with [17], who mention in the PCR, *exo*A gene was detected in 57 isolates 81.5% out of 70 isolates of *P. aeruginosa,* while differ with Sana'a, the researcher found the isolates was 57% harbored this gene [18]. It was demonstrated that *opr*D gene with a band size of 1329-bp was detected in 9 isolates 90% out of 10 isolates of *P. aeurginosa,* but was not detected in 1 isolate this result similar to our study [19]. On the other hand, the current study differed with previous studies, [20], which pointed that the PCR detected *opr*D gene in 38 isolates 100% isolates of *P. aeruginosa* were isolated from a different clinical specimens.

Antimicrobial Susceptibility Test of P. Aeruginosa

A total of 12 antimicrobial agents, which belongs to 4 families of antibiotics were used in this study (8). The results of the current study shown in the table(3) indicated that from all *P. aeruginosa* 12 isolates showed many antibiotics resistance which include: Imipenim 50%, Meropenem 41.7%, Ceftazedim 25%, Cefipim 75%, Levofloxacin 58.3%, Norfloxacin 41.7%, Ciprofloxacin 50%, Gentamycin 58.3%, Nitlimicin 50%, Azetreonam 75%, Piperacillin/Tazobactam 66.7% and Ticarcillin/Clavulanate 66.7% . Isolates showed complete sensitivity to Imipenem which was similar with study of [21], who indicated 50%. Pipracilline/Tazobactam was disagree with [21], who indicated 50%. [22], pointed that the resistant rates for Levofloxacin was 40%, this result was disagree with present study. [23], was showed that the bacteria was completely sensitive 100% to Levofloxacin, this result was disagree with present study. The percentage of resistant for Imipenem was 50% this finding was disagree with [24], who reported 5%. When comparing the present results with AL-Janabi, the researcher found the resistance of *P. aeruginosa* isolates against Ciprofloxacin, Gentamicin, Piperacillin/Tazobactam and Impenem were 26%, 30% ,

48% and 13.3% respectively [25], these results were variable with present study. Another studies in Iraq by [26], the percentage of resistan for Ciprofloxacin was 80%, these results were disagree to the present study. The variable in resistance may be due to the sources of samples and ecological and test conditions.

It was concluded from this study and previous studies that the cause of Antibiotic resistance in *P. aeruginosa* is caused by a variety of mechanisms, including the ability to alter membrane permeability. It also owns Efflux pumps and manufactures wide-narrow betalactimase enzymes and biofilm formation, as well as R-resistance plasmids containing various antibiotic resistance genes [27]. The pathogenicity of *P. aeruginosa may* be aided by a number of virulence factors. By using PCR to identify clinical *P. aeruginosa,* the *opr*D and toxA genes were assessed to see whether there was a correlation between particular virulence factors and the distinct manifestations of *P. aeruginosa* infections, virulence factors, which were identified by PCR in these isolates [28].

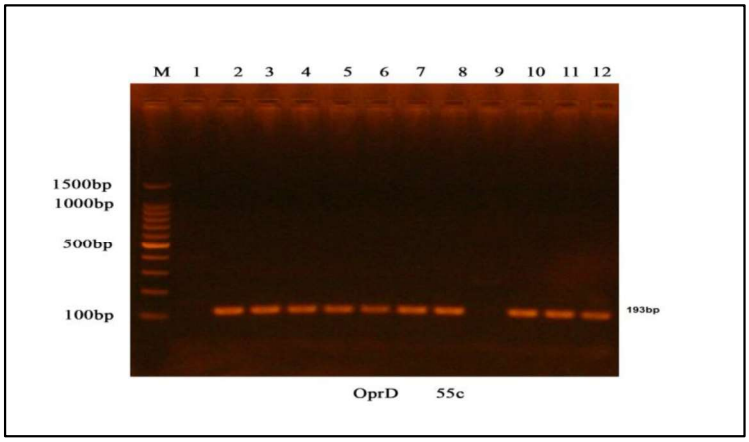

FIGURE 1. Gel electrophoresis of *opr*D in *P. aeruginosa* in 1X TAE buffer using 100 V. for approximately 75min. Lane1:100bp kappa universal ladder; lanes 1,9:negative results; lanes 2,3,4,5,6,7,8,10,11.12 : 193bp of *Opr*D PCR product.

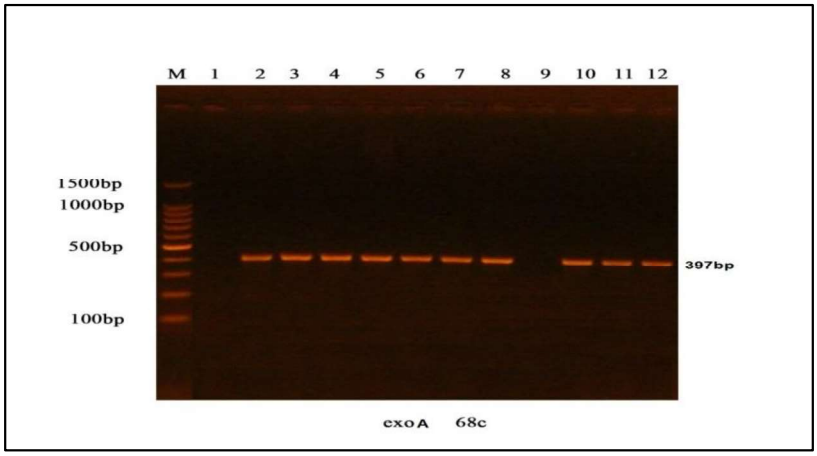

FIGURE 2. Agarose gel (1.5%) electrophoresis of *exo*A PCR product for *P. aeruginosa* in 1X TAE buffer using 100 V. for approximately 75min. Lane1:100bp kappa universal ladder; lanes 1,9:negative results; lanes 2,3,4,5,6,7,8,10,11.12 : 397bp of *Exo*A PCR product.

TABLE 2. Antibiotics sensitivity of 12 antibiotics against 12 *P. aeruginosa* isolates.

NO.	ATM	PTZ	TIM	CAZ	CPM	MEM	IMI	CIP	LEV	NOR	GM	NET
P1	R	R	R	R	R	R	R	R	R	R	R	R
P2	R	R	R	S	R	R	S	R	R	R	R	R
P3	S	S	S	S	S	S	S	S	S	S	S	S
P4	S	S	R	S	S	S	S	S	S	S	S	S
P5	R	S	S	S	R	S	R	S	R	S	S	S
P6	R	R	S	S	R	S	R	S	S	R	S	S

NO.	ATM	PTZ	TIM	CAZ	CPM	MEM	IMI	CIP	LEV	NOR	GM	NET
P7	R	R	S	S	R	S	S	R	R	S	R	S
P8	R	R	R	S	R	S	S	S	S	S	R	R
P9	R	R	R	R	R	R	R	R	R	R	R	R
P10	R	R	R	R	R	R	R	R	R	S	R	R
P11	S	S	R	S	S	S	S	S	S	S	S	S
P12	R	R	R	S	R	R	R	R	R	R	R	R

ATM=Azetreonam, PTZ=Piperacillin/Tazobactam, TIM=Ticarcillin/Clavulanate, CAZ=Ceftazedim, CPM=Cefipim, MEM=Meropenem, IMI=Imipenim, CIP=Ciprofloxacin, LEV=Levofloxacin, NOR= Norfloxacin, GM= Gentamycin, NET= Nitlimicin.

TABLE 3. The resistant rate of the *P. aeruginosa* against different antibiotics.

Antibiotics	NO. of S/12	NO. of R/12	Resistant rate
ATM	3	4	75%
PTZ	4	8	66.7%
TIM	4	8	66.7%
CAZ	9	3	25%
CPM	3	9	75%
MEM	7	5	41.7%
IMI	6	6	50%
CIP	6	6	50%
LEV	5	7	58.3%
NOR	7	5	41.7%
GM	5	7	58.3%
NET	6	6	50%

CONCLUSION

This research indicates that molecular detection of P. aeruginosa from clinical samples employing oprD and exoA genes by using PCR technique is a rapid and easy method for P. aeruginosa infection diagnosis, which have higher sensitivity and specificity. It was shown that the results of the PCR assay on clinical samples of severe infections became positive much earlier than the results of conventional culture method. In addition, it was detected that oprD and exoA play a major role in the antibiotic sensitivity of this bacterium.

REFERENCES

1. J. Klockgether and B. Tümmler “Recent advances in understanding Pseudomonas aeruginosa as a pathogen,” F1000Research, vol. 6, 2017.
2. C. K. Stover, X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey and M. V. Olson “Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen,” Nature, vol. 406, no. 6799, pp. 959-964, 2000.
3. M. F. Moradali, S. Ghods and B. H. Rehm “Pseudomonas aeruginosa lifestyle: a paradigm for adaptation, survival, and persistence,” Frontiers in cellular and infection microbiology, vol. 7, pp. 39, 2017.
4. P. Baker, P. J. Hill, B. D. Snarr, N. Alnabelseya, M. J. Pestrak, M. J. Lee and P. L. Howell “Exopolysaccharide biosynthetic glycoside hydrolases can be utilized to disrupt and prevent Pseudomonas aeruginosa biofilms,” Science advances, vol. 2, no. 5, pp. e1501632, 2016.
5. A. Lorenz, M. Preuße, S. Bruchmann, V. Pawar, N. Grahl, M. C. Pils and S. Häussler “Importance of flagella in acute and chronic Pseudomonas aeruginosa infections,” Environmental microbiology, vol. 21, no. 3, pp. 883-897, 2019.
6. F. Abbasloo, S. A. Khosravani, M. Ghaedi, K. Dashtian, E. Hosseini, L. Manzouri and F. Sadri “Sonochemical-solvothermal synthesis of guanine embedded copper based metal-organic framework

(MOF) and its effect on oprD gene expression in clinical and standard strains of Pseudomonas aeruginosa," Ultrasonics sonochemistry, vol. 42, pp. 237-243, 2018.
7. Hossein, H.M.; Mehdi, R. M.; Masoumeh, A.; Gholamreza, A. and Masoud, D.M. (2015). Molecular evaluation of pseudomonas aeruginosa isolated from patients in burn ward, ICU and ITU in a number of hospital in Kerman province 5 (S2): 1428-1431.
8. The clinical and laboratory standards institute CLSI (2020).
9. Sara and Anfal (2021) Evaluation of Biofilm Formation in Klebsiella Pneumoniae andAntibiotic Resistance. Indian Journal of Forensic Medicine & Toxicology, 2021, Vol. 15, No. 2 3413.
10. R. G. Brennan, S. A. Rabb, M. J. Holden, M. R. Winchester and G. C. Turk "Potential primary measurement tool for the quantification of DNA," Analytical chemistry, vol. 81, no. 9, pp. 3414-3420, 2009.
11. A. Van Belkum, G. Durand, M. Peyret, S. Chatellier, G. Zambardi, J. Schrenzel and W. M. Dunne "Rapid clinical bacteriology and its future impact," Annals of laboratory medicine, vol. 33, no. 1, pp. 14-27, 2013.
12. D. Rodríguez-Lázaro and M. Hernández "Real-time PCR in food science: introduction," Current issues in molecular biology, vol. 15, no. 2, pp. 25-38, 2013.
13. D. De Vos, C. Bouton, A. Sarniguet, P. De Vos, M. Vauterin and P. Cornelis "Sequence diversity of the oprI gene, coding for major outer membrane lipoprotein I, among rRNA group I pseudomonads," Journal of bacteriology, vol. 180, no. 24, pp. 6551-6556, 1998.
14. A. A. Khan and C. E. Cerniglia "Detection of Pseudomonas aeruginosa from clinical and environmental samples by amplification of the exotoxin A gene using PCR," Applied and environmental microbiology, vol. 60, no. 10, pp. 3739-3745, 1994.
15. J. Weile and C. Knabbe "Current applications and future trends of molecular diagnostics in clinical bacteriology," Analytical and bioanalytical chemistry, vol. 394, no. 3, pp. 731-742, 2009.
16. K. Wolska and P. Szweda "Genetic features of clinical Pseudomonas aeruginosa strains," Pol J Microbiol, vol. 58, no. 3, pp. 255-60, 2009.
17. S. R. Taee, B. Khansarinejad, H. Abtahi, M. Najafimosleh and E. Ghaznavi-Rad "Detection of algD, oprL and exoA genes by new specific primers as an efficient, rapid and accurate procedure for direct diagnosis of Pseudomonas aeruginosa strains in clinical samples," Jundishapur journal of microbiology, vol. 7, no. 10, 2014.
18. N. H. Sana'a "Detection of Exo A and OPR I Genes in Pseudomonas aeruginosa Using Polymerase Chain Reaction," Iraqi Journal of Biotechnology, vol. 12, no. 1, pp. 44-50, 2013.
19. E. E. Nmema, C. S. Osuagwu and E. N. Anaele "oprD Genes Detected in Pseudomonas aeruginosa Isolates from a Teaching Hospital but Lost in a Carbapenem-Resistant Strain," Journal of Advances in Medicine and Medical Research, pp. 1-8, 2019.
20. A. B. Al-Hashimy, H. S. Alagely, A. K. Albuaji and K. R. Majeed "Identification Pseudomonas aeruginosa by OprD Gene for Differentiation from Other Pseudomonas Species that Isolated from Clinical Samples," International Journal of Drug Delivery Technology, vol. 9, no. 1, pp. 46-50, 2019.
21. L. Krishnasamy, C. Saikumar and G. Kumaramanickavel "Aerobic Bacterial Pathogens Causing Vaginitis in Patients Attending A Tertiary Care Hospital and their Antibiotic Susceptibility Pattern," J Pure Appl Microbiol, vol. 13, no. 2, pp. 1169-1174, 2019.
22. Akingbade ,O.A.; Balohun, S.A.; Ojo, D.A.; Afolabi, R.O.; Motayo, B.O.;Okerentugba,P.O. and Okonko,I.O .(2012) .Plasmid profile analysis of multidrug resistant Pseudomonas aeruginosa isolated from wound infections in South West ,Nigeria .World Applied Sciences Journal .20(6) :766-775.
23. Al–Shammari, H.A.A. (2016). Detection of Some Virulence Factors in Pseudomonas aeruginosa Isolated from Different Sources. Master thesis. College of Medicine, Babylon University.
24. Hasan, S. A.;Najati, A. M. and Abass, K. S. (2020). „Prevalence and antibiotic resistance of "pseudomonas aeruginosa" isolated from clinical samples in Kirkuk City, Iraq", EurAsian Journal of BioSciences. Foundation for Environmental Protection and Research, 14(1), pp. 1821–1825.
25. AL-Janabi,T.Y.S. (2019).Biosynthesis of Zinc Oxide Nanoparticles by Pseudomonas aeruginosa Locally Isolated.Master Thesis. College of science /Baghdad university.
26. A. M Hamdoon, A. Z Al-Gerir and H. M Al-Habib "Profile of Pseudomonas aeruginosa in burn infection and their antibiogram study," Annals of the College of Medicine, vol. 37, no. 1, pp. 57-65, 2011.
27. J. S. Hong, E. J. Yoon, H. Lee, S. H. Jeong and K. Lee "Clonal dissemination of Pseudomonas aeruginosa sequence type 235 isolates carrying bla IMP-6 and emergence of bla GES-24 and bla IMP-10 on novel

genomic islands PAGI-15 and-16 in South Korea," Antimicrobial agents and chemotherapy, vol. 60, no. 12, pp. 7216-7223, 2016.
28. V. S. Nikbin, M. M. Aslani, Z. Sharafi, M. Hashemipour, F. Shahcheraghi and G. H. Ebrahimipour "Molecular identification and detection of virulence genes among Pseudomonas aeruginosa isolated from different infectious origins," Iranian journal of microbiology, vol. 4, no. 3, pp. 118, 2012.