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Research Article

Identification *Pseudomonas aeruginosa* by *OprD* Gene for Differentiation from Other Pseudomonas Species that Isolated from Clinical Samples

Ashwak B Al-Hashimy[1], Khalid R Majeed[2], Akeel K Albuaji[1*], Huda S Alagely[1]

[1]*Institute of genetic engineering and biotechnology for postgraduate studies, university of Baghdad, Iraq.*
[2]*Department of Medical Laboratory Techniques /Technical Institute Nasiriyah /Southern Technical University/Iraq.*

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ABSTRACT
The present study included the collection of 100 samples from various clinical sources for investigating the presence of *P. aeruginosa* in those sources, the samples have been collected from some hospitals in Baghdad and Hillah city (Al-qassim General Hospital, ,Al-hillah teaching hospital,and Al-hashimya General hospital) which included wounds, burns, ear and sputum infections. The study was carried out through October 2017 till the end of March 2018. The samples were identified based on the morphological and microscopically characteristics of the colonies when they were culturing or number of culture media as well as biochemical tests, molecular identification were also used as a final diagnostic test for isolates that were positive as they belong to *P.aeruginosa* bacteria during previous tests based on the *OprD* gene which has specific sequences for *P.aeruginosa* bacteria as a detection gene and also consider as virulence factor so it have a synonyms mechanism to antibiotic resistance . The results of the final diagnosis showed that 38 isolates belong to target bacteria were distributed as 18 of burns, 11 isolates of wounds, 6 isolates of ear infection and 3 isolates of sputum, The examination of the sensitivity of all bacterial isolates was done for elected 38 isolation towards the 9 antibiotic by a Bauer - Kirby and the isolates were resistant for a number of antibiotics used such as Ciprofloxacin 65.7%, Norflaxacin 71%, Imipenem 63.1% Meropenem 68.4%, Gentamicin 65.7%, Amikacin 26.3%, Cefepime 68.4%, Ceftazidime 65.7% and Piperacillin 57.8%.Molecular method , All isolates (38) of *P. aeruginosa* positive for the diagnostic special gene (*OprD*) genes (100%).

Keywords: *Pseudomonas aeruginosa, OprD*, mutations, isolates.

INTRODUCTION

Pseudomonas aeruginosa is an aerobic gram negative bacterium which has emerged as one of the most problematic nosocomial pathogen to characterizes *p. aeruginosa* strains that are widespread in Iraqi patients[8]. Multidrug-resistant (MDR) *P. aeruginosa* is difficult to eradicate due to elevated intrinsic resistance and its ability to acquire resistance to multiple antibiotics[4]. This nosocomial pathogen has various mechanisms of resistance to antibiotics, such as broad spectrum ß-lactamases and metallo-ß-lactamases (MBL), through the alteration of penicillin-binding proteins (PBP), porin mutations, plasmid enzymatic modification, DNA-gyrase mutations, and active pumps[1]. *Pseudomonas aeruginosa* is a clinically significant opportunistic pathogen which rarely causes disease in healthy immune competent individuals, The emergence of multi-drug resistant strains in *P. aeruginosa* isolates has increased worlwide. Fluoroquinolones act as bactericidal agents by inhibiting DNA gyrase and topoisomerase IV, thus inhibiting DNA transcription and replication[3].

Fluoroquinolon was originally developed as an excellent activity against aerobic Gram-negative bacteria, while its activity against Gram-positive organisms is limited. Ciprofloxacin, noroflaxacin, enofloxacin, lomefloxacin, levofloxacin, ofloxacin are agents that have activity both Gram-negative and of moderate to good activity against Gram-positive bacteria. In this group, ciprofloxacin is the most active agents against Gram-negative, especially *P. aeruginos*[13,15]. Among the several mutation-mediated resistance mechanisms resistance to carbapenems. existing in *P. aeruginosa* are those conferring decreased susceptibility or resistance to flouroquinolone outer membrane protein (OMP) OprD, a specialized porin which has a specific role in the uptake of positively charged amino acids such as lysine and glutamate[12].

MATERIAL AND METHOD

Clinical isolates

A total of (100) clinical specimen were collected from the beginning of October 2017 to the end of January 2018. from patients seeking medical advice in (Al-qassim General Hospital, Al-Kindi teaching hospital and Al-hillah teaching hospital,and Al-hashimya General hospital)taken from patients' burn (40), wound (30), ear swab (25) and sputum (5).

Isolation and identification of pseudomonas aeruginosa by Traditional methods

Author for Correspondence akeel654albuaji@yahoo.com

Table 1: The Sequence Forward and Reverse Primer of *0prD* .

Genes	Primer Sequence (5' to 3')	Size (bp)	References
oprD	F:TGCTGCTCCGCAACTACTATTTC R:GTAGGCCAAGGTGAAAGTGTG	752	(5)

Figure 1: Biochemical identification of *pseudomonas aeruginosa* using API 20E strep.

Table 2: The Mixture of Conventional PCR working solution for Detection *OprD* Gene in *Pseudomonas aeruginosa.*

Component	Concentration	Volume (µl)
Master mix	2x	Lyophilized
Primer F.	10 pmol	1
Primer R.	10 pmol	1
DNA	50 ng	5
Deionizer water	---	13
Total Volume	---	20µl

Figure 2: *Pseudomonas aeruginosa* culture on Cetrimide Agar.

The isolates were identified by means of routine tests: colony morphology and pigment formation on selective medium (cetrimide agar), catalase test, oxidase reaction, non glucose fermentation, growth at 42 °C, and identify by Api 20E.fig(1)[7,8].

Molecular identification of pseudomonas aeruginosa

Bacterial Genomic DNA Extraction

DNA was extracted from activated pure culture of *P.aeruginosa* bacteria using Presto™ Minig DNA

Table 3: PCR Program for *OPr*D, Gene Amplification by Conventional Methods.

No.	Steps	Temperature (°C)	Time	Cycles
1	Initial Denaturation	94	5min	1
2	Denaturation	94	45 sec	35
3	Annealing	54	30sec	
4	Extension	72	1min	
5	Final extension	72	4min	1

Bacteria Kit (DisBIO) Purification depending on instruction of manufacturing company (DisBIO, China).

Detection of pseudomonas aeruginosa by molecular method

The culture was stored at -20C°prior to PCR amplification and detection of DNA bands using Agarose gel electrophoresis (1%)[10,11] Primer selection**.** The primers used in this study are shown in (Table-1). PCR amplification of outer membrane porin (*OprD*) for the detection of *Pseudomonas aeruginosa*.

PCR amplification

All reaction mixtures were set up in a PCR room separate from that used for DNA extraction and amplification PCR was completed in adapted PCR micro centrifuge tubes according to the thermocycler used. Following optimization, reaction mixtures (20 µl) were set up as follows: 13µl deionizer water, PCR master mix (Wizbio) ,(2µl F+R) of each set of primer (*OprD* gene) and 5 µl of DNA template (table- 2). The reaction mixtures were subjected to the following empirically optimized thermal cycling parameters in a thermocycler (Senso-Quest Labcycler, Germany): 94°C for 5 min, followed by 35 cycles of 94°C for 45 sec, 54°C for 30 sec, 72°C for 1min, and a final extension at 72°C for 4 min (14) (table-2).

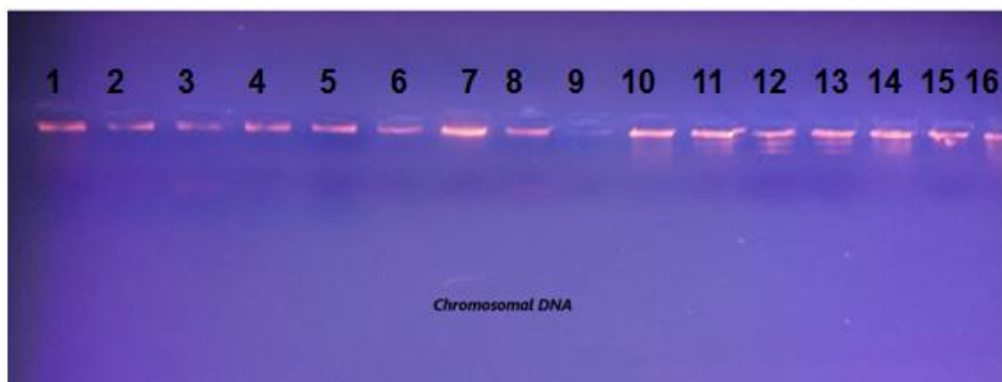

Figure 3: Gel electrophoresis of DNA extraction of *P. aeruginosa* isolates using 1% Agarose for 45 min. at 75 volt.

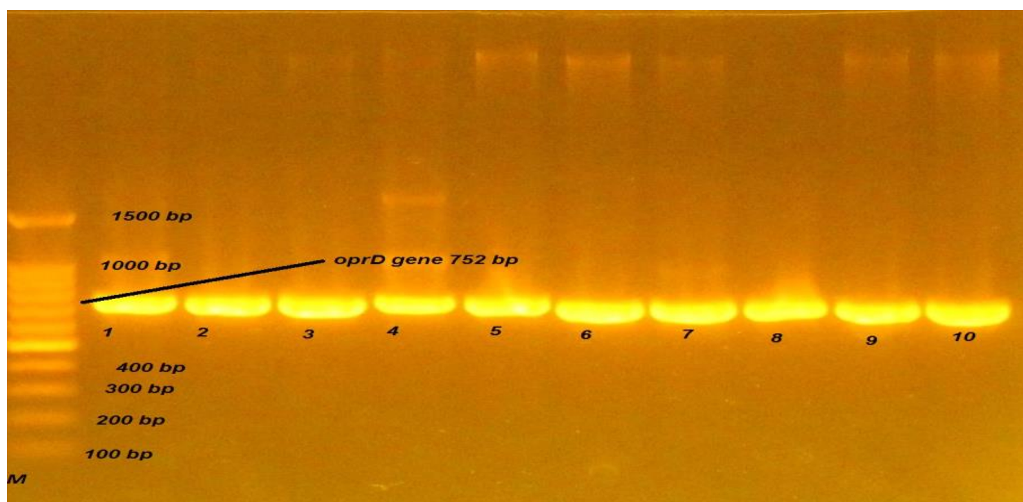

Figure 4: Agarose gel electrophoresis (1% agarose, 100Vol / 1.20 hour) of PCR amplification products of *P.aeruginosa oprD*752bp Lanes 2-10 : show positive.

Following amplification, aliquots (5µl) were removed from each reaction mixture and examined by means of electrophoresis (75 V,1.40 min) in gels composed of 1% agarose in TBE buffer (40 mM Tris, 20 mM boric acid,1 mM EDTA, pH 8.3). Gels were visualized under UV illumination by using a gel image analysis system (UVitec, Cambridge, United Kingdom) (2)(6) and all images were archived. Where a band was visualized at the correct expected size for *OprD*, the specimen was considered positive for *Pseudomonas* genus; if a band was visualized at the correct expected size for *OprD* loci, the specimen was considered positive for *P. aeruginosa* figure (4).

Antimicrobial susceptibility

The drug susceptibility test was carried out for all the isolateson Mueller-Hinton plates disk diffusion method was used to measure zones of inhibition in accordance with the recommendations of clinical and laboratory standards institute (6). The test antibiotics were:

Ciprofloxacin (5µg) Noroflaxacin (10%µg), Imipinem (10%µg), Meropinem (30µg) Gentamicin (10 µg), Amikacin (30 µg), Cefepime (30 µg), Ceftazidime (10 µg), Pipercillin (30µg).

RESULT AND DISCUSSION

A total of 100 different clinical specimens were collected from October 2017 to the end of January 2018, 38 isolates were collected from different specimens include wounds, burns, ear swabs, and sputum. in five hospitals in Baghdad and hillah .from both gender with age ranged from 5year to 75 year. It was found that most common causes of burns All colonies appear mucoid, smooth in shape with flat edges and elevated center, creamy color and have a fruity odor on cetrimide agar fig. (1). In another hand all 38 isolates gave positive result for oxidase, catalase and did not produce H_2S. figure (1).

Total DNA was extracted using Presto Mini g DNA bacteria Kits with concentration 53ng and purity between 1.7- 1.9 then detected by gel electrophoresis as show in fig.(2).

PCR techniques were used for further conformational diagnosis of all (38) isolates of *Pseudomonas* bacteria, depending on species specific primer for the *OprD* gene, which are specific for diagnosis of *Pseudomonas aeruginosa* (752bp) this result agreement with (10)(11) , which give same result when compare with biochemical test (API 20 strep) which is specific for diagnosis of *P.aerugenosa*, product of PCR detect by using gel electrophoresis as show in figure (3). Clear correlation was appeared between PCR techniques and culturing method.

Table 4: Antibiotic Susceptibility patterns of *P. aeruginosa* isolates associated with clinically significant burn wound, ear and sputum infection by Bauer - Kirby methed.

Antibiotics	Sensitive N%		Intermediate N %		Resistant N%	
Ciprofloxacin(CIP)	11	28.9	2	5.2	25	65.7
Norflaxacin (NOR)	10	26.3	1	2.6	27	71
Imipenem (Ipm)	12	31.5	-	-	24	63.1
Meropenem(MEM)	11	28.9	1	2.6	26	68.4
Gentamicin (GM)	11	28.9	2	5.2	25	65.7
Amikacin(AK)	17	44.7	1	2.6	10	26.3
Cefepime (FEP)	10	26.3	4	10.5	24	68.4
Ceftazidime(CAZ)	9	23.6	4	10.5	25	65.7
Piperacillin (PIP)	10	26.3	6	15.7	22	57.8

Antimicrobial sensitivity test were conducted for 38 isolates of testing of the 38 isolates of *P.aeruginosa* using 9 types of antibiotics used in the hospitals .According to the results obtained in the Table (4).

Ciproflaxacin (65.7%), Norflaxacin (71%), Impenem (63.1%), Meropenem (68.4%) Gentamicin (65.7%), Amikacin (26.3%), Ceftazidime (65.7%), Cefepime 68.4% and pipercillin (57.8%).

The *P. aeruginosa* family of porin proteins, defined based on their apparent sequence homology within the *P. aeruginosa* genome, plays an important physiological role in the transport of substances required for metabolism. However, these proteins also exhibit an affinity for certain hydrophilic antibiotics, such as β-lactams, aminoglycosides, tetracyclins, and some fluoroquinolones, allowing these compounds to transverse the otherwise insoluble outer bacterial membrane[15]. Deletion of one or more porin proteins has been shown to reduce the susceptibility of *P. aeruginosa* to certain antibacterial agents[16].

The *P. aeruginosa* porin OprD is a substrate-specific porin that facilitates the diffusion of basic amino acids, small peptides, and carbapenems into the cell[17]. OprD-mediated resistance occurs as a result of decreased transcriptional expression of *oprD* and/or loss of function mutations that disrupt protein activity. Specific mechanisms resulting in decreased transcriptional expression of *oprD* include (i) disruption of the *oprD* promoter, (ii) premature termination of *oprD* transcription, (iii) co-regulation with trace metal resistance mechanisms, (iv) salicylate-mediated reduction, and (v) decreased transcriptional expression via co-regulation with the multidrug efflux pump encoded by *mexEF-oprN*[18].

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