British Journal of Pharmacology and Toxicology 1(2): 85-89, 2010
ISSN: 2044-2467

Submitted date: August 25, 2010 Accepted date: September 28, 2010 Published date: November 15, 2010

Genetic Site Determination of Antibiotic Resistance Genes in *Pseudomonas aeruginosa* by Genetic Transformation

[1]K.K. Adel and [2]S.S. Sabiha
[1]Biology Department, College of Education/Scientific Departments,
University of Salahadeen, Erbil, Iraq
[2]Technical Agricultural Institute, Sulaymani, Iraq

Abstract: To determine the site of antibiotic resistant genes in *Pseudomonas aeruginosa*, 50 *P. aeruginosa* were isolated out of 250 samples from hospitalized patients in different hospitals in Sulaymani city, Iraq, more over isolated from soil dustbin and sewage water. The isolates were identified according to the cultural, morphological and biochemical tests, in addition to API 20E system. The isolated bacteria were screened for their resistance to seventeen commonly used antibiotics. The isolated *P. aeruginosa* show different pattern of antibiotic resistance, they grouped in to 34 antibiotype. Organisms were 100% resist to (Amp, Cef, Er, Gm, Lin, Pip and Cln), the lowest resistant recorded was 35% for Rif. Six *P. aeruginosa* isolates 12% revealed resistant to all seventeen used antibiotics, while nine isolates 18% were sensitive to one antibiotic, and 10 isolates were sensitive to two antibiotics, and remaining 50 isolates were sensitive to 3-5 antibiotics. Transformation process appeared that the genes responsible for all tested antibiotics except Lin in P1 isolate, and Lin and Tob in P8 isolate are located on plasmid DNA in isolated *P. aeruginosa*.

Key words: Antibiotics, chromosomal DNA, multi-drug resistance, transformation, plasmid, *Pseudomonas aeruginosa*

INTRODUCTION

Antibiotic resistance is recognized as being one of the major medical problems facing humankind and measures to prevent, or contain, the increase in antibiotic resistant organisms requires knowledge of both the genesis of antibiotic resistance genes and their dissemination. The problem of antibiotic resistance affects almost every bacterial species for which treatment with antibiotics is available (Salih, 2008).

Pseudomonas aeruginosa is the epitome of an opportunistic pathogen of human. The bacterium it causes urinary tract infection, respiratory infection, dermatitis, soft tissue infections, bacteremia, bone and Joint infections, gastrointestinal infections and variety of systemic infection, particular in patients with sever burns and in cancer, AIDS patients who are immune-suppressed and other diseases it causes are pneumonia, endocarditic, chronic lung infections and septicemia. The bacterium is the 4th most commonly isolated nosocomial pathogen account for 10.1% of all nosocomial infections (Nelson and Reginald, 2007).

Plasmids are most replicating circular pieces of DNA, smaller than the bacterial genome which encoded their transfer by replication into another bacterial strain or species. They can carry and transfer multiple resistance genes, which may be located on section of DNA capable of transfer from one plasmid to another or to the genome-transposon or jumping gene (Snyder and Champness, 1997).

Plasmid in *P. aeruginosa* the pathogenic nature and its resistant to most antibiotics and the success of this species as common hospitalized pathogen organisms, lead to many studies on this organism including their plasmids, which are responsible for the inactivation of antibiotics (Khider, 2002). Researchers had extracted many R-plasmids that confer resistance to a number of antibiotics such as penicillin, rifampicin (Jawetz *et al*., 2001). It was found that most of these plasmids are transferable to either other bacteria or other genus, with different molecular weight. Plasmids play a significant role in bacterial adaptation and evolution.

This study describes the resistance to drugs of *P. aeruginosa* isolated from different environments, and determination the site of antibiotic resistant genes in isolated bacteria.

MATERIALS AND METHODS

Test organism confirmation: A total of 150 samples were taken from human infections collected from the microbiology laboratory unit of Sulaymani Teaching

Corresponding Author: K.K. Adel, Biology Department, College of Education/Scientific Departments, University of Salahadeen, Erbil, Iraq

Hospital Sulaymani, Iraq during January to April 2007. Similarly a few tests were carried out to reconfirm the test organisms including gram staining, catalase test, Oxidase test and motility (Morello *et al*., 2003). Moreover the API20E (Bio Merieux, Marcyl, Etoile, France) system was performed. The pure cultures were sub cultured on nutrient agar slants and preserved in the refrigerator at 4ºC until required for the study.

Determination of antibiotic susceptibility: The antimicrobial resistance phenotypes of all isolated bacteria were determined using dilution method in agar plate (Atlas *et al*., 1995). The antibiotics purchased from sigma company Germany are Clindamycin (Cln), Ampicillin (Amp), Cefotaxime (Cef), Erythromycin (Er), Gentamycin (Gm), Lincomycin (Lin), Penicillin (Pn), Trimethoprim (Tri), Tetracycline (Tet), Tobramycin (Tob), Pipracillin (Pip), Chloramphenicol (Chl), Striptomycin (Str), Nitrofuration (Nit), Ciprofloxacin (Cip), Nalidixic acid (Nal), Rifampicin (Rif). These antibiotics were used at final concentrations were added to the medium after sterilization and Cooling to 50ºC, the medium were mixed and poured into Petri-dishes, then inoculated using streaking method, resistance were recorded after incubation for 24 h at 37ºC.

Plasmid preparation: This method, used for the screening of large number of transformants, was described by (Birnboim and Doly, 1979; Kochonic *et al*., 2003). A 105 mL aliquot of a bacterial culture grown in selective media was pelleted at 4000 Xg. The pellet was resuspended by vortexing in 200 µL of solution No. 1, and then left for 10 minutes at room temperature. Then 200 µL of solution No. 2 was added and the tube was mixed by inversion and placed on ice for 5 min. Then 200 µL of solution No. 3 was added and the tube was mixed by inversion and placed on ice for 10 min. The supernatant was placed into fresh tube and 400 µL of phenol-chloroform isoamylealcohol (25:24:1) was added and mixed. After centrifugation at 13,000 Xg for 5 min the aqueous layer was removed to fresh tube and an equal volume of isopropanol was added. After 10 min at room temperature the tube was centrifuged for 10 min at 13,000 X g to pellet the plasmid DNA. The pellet was washed with 70% ethanol, dried briefly in a vacuum dryer and resuspended in 50 µL of TE buffer. Plasmid prepared was stored at 4ºC.

Preparation of competent cells: A modified method of Sambrook *et al*. (1989) was used for preparation of competent cells for transformation. *E. coli* DH5alpha cells. An overnight culture of *E. coli* was prepared by suspending a colony from a fresh nutrient agar plate in 100 ml nutrient broth. The culture was incubated in a shaking incubator at 100 rpm at 37ºC for 24 h. 10 mL of this culture was then suspended in 90 mL fresh nutrient broth and grow for 90 min to an OD 600 nm of approximately 0.3. Ten ml aliquots were centrifuged at 4000 X g for 10 min to pellet the cells. The supernatant was discarded and the cells were washed in 5 mL ice-cold 10 mM NaCl and re-countergauged. The supernatant was discarded and cells were resuspended in $CaCl_2$ and recenterifuged, twice. Following a third washing with 5 mL 30 mM $CaCl_2$, cells were then recenterfuged and resuspended in 1 mL of ice-cold 30 mM $CaCl_2$ +15% glycerol. The cells could be used immediately for transformation or stored at -20ºC.

Transformation: Two hundred µL of *E. coli* competent cells were mixed with 2 µL of extracted plasmid, the tubes then left on ice for 30 min then incubated for 30 sec at 42ºC in a water bath and placed on ice for a further 5 min. 800 µL of nutrient broth was added and the tubes were incubated at 37ºC, then the cells were centrifuged at 6000 X G for 1 min. the pellet was resuspended in 200 µL of nutrient broth, 100 µL of the cells were plated out on nutrient agar containing appropriate antibiotic markers. Post incubation, the plates were screened for recombination, which were selected and streaked onto fresh agar plates for plasmid purification (Ausabel *et al*., 2003).

Agarose gel preparation: Agarose gel 0.8% was prepared in 100 mL 1 X TE buffer and boiled, this was allowed to cool to 50ºC and 2 µL (10 mg/mL) of ethidium bromide was added. The gel was poured and comb was inserted to make the walls. When the gel was set, the comb was removed and the gel was placed in the gel box and immersed in 1 X TAE buffer. Samples were prepared by taking 10 µL of the DNA, 2 µL of loading dye. The gels were run for 90 min at 75 Volt, the gels were viewed and photographed (Ausabel *et al*., 2003).

RESULTS

It was of interest to study clinical isolates of *P. aeruginosa* obtained from different Hospitals in Sulaymani city, Iraq. Preliminary identification tests were performed on all isolates (Gram stain, oxidase, catalase and motility). The isolates were identified using a variety of techniques. These include morphological characteristics, Biochemical testes, pigment production and API 20E system was performed.

The cells and Varity of biochemical tests of the isolates are indicated that all isolates were identified as being G^-, non spore forming, motile rods. The isolates were all oxidase and catalase positive and were shown to be oxidative. The isolates also were all ß-haemolytic and grow well at both 37 and 42ºC, but were unable to grow at 4ºC. All isolates produced yellow/green pigment

Table 1: Antibiotics used, number of resistant isolates and % of resistance of *P. aeruginosa* isolates

Antibiotics	No. of resistant isolates	Resistance (%)
Cln	50	100
Amp	50	100
Cef	50	100
ER	50	100
Gm	50	100
Lin	50	100
Pn	50	100
Tri	49	98
Tet	48	96
Tob	45	90
Pip	45	90
Chl	41	80
Str	37	74
Nit	36	72
Cip	33	67
Nal	33	67
Rif	17	35

indicates of fluorescein pigment production, blue/green indicative of the pyocyanin production. At the basis of these results and according to (Holt *et al*., 1994), the bacteria were identified as *P. aeruginosa*. Moreover all of the isolates were identified as *P. aeruginosa* by API 20E identification Kit.

Fifty *P. aeruginosa* isolates were screened for their resistance to 17 widely used antibiotics in medicine. Table 1 illustrates that all isolates vary in their responding to the use of the antimicrobials agents, showed that all of examined isolates were resistant 100% to (Amp, Cef, Er, Gm, Lin, Pn and Cln), while the lowest percentage of resistance was 35% to (Rif). The isolated bacteria were grouped in to 34 antibiotype, and the resistant pattern is shown in Table 2. Among 50 isolated *P. aeruginosa* 6 of them 12% show resistance to all seventeen used antibiotics group (2), and 9 isolates 18% were sensitive to

Table 2: Antibiogram groups of *P. aeruginosa* isolates

Antibiogram group	No. of isolates	Serial number of isolates	Antibiotics																
			Amp	Cef	Chl	Cip	Er	Nit	Gm	Lin	Nal	Pn	Pip	Rif	Tet	Tri	Tob	Str	Cln
1	1	P1	+	+	+	-	+	-	+	+	-	+	+	+	+	+	-	-	+
2	6	P2,P8,P20, P6,P28,P29	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	1	P3	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+
4	1	P4	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	-	+
5	1	P5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
6	1	P7	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+
7	1	P9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
8	1	P10	+	+	+	+	+	-	+	+	+	+	-	-	+	+	+	-	+
9	2	P11,P12	+	+	+	-	+	-	+	+	-	+	+	-	+	+	+	-	+
10	3	P13,P15,P47	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+
11	2	P27,P14	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
12	1	P16	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	-	+
13	1	P17	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+
14	1	P18	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+
15	1	P19	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+
16	2	P41,P21	+	+	+	-	+	+	+	+	+	+	-	-	+	+	+	-	+
17	3	P22,P25,P30	+	+	+	-	+	-	+	+	+	+	+	-	+	+	+	+	+
18	1	P23	+	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+	+
19	1	P24	+	+	+	-	+	+	+	+	-+	+	+	-	+	+	+	-	+
20	5	P26,P34,P31, P49,P50	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
21	1	P32	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+
22	1	P33	+	+	-	-	+	+	+	+	-	+	+	-	+	+	+	+	+
23	1	P35	+	+	+	-	+	+	+	+	-	+	+	-	-	+	+	+	+
24	1	P36	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
25	1	P37	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+
26	1	P38	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+
27	1	P49	+	+	-	-	+	-	+	+	+	+	+	-	+	+	+	+	+
28	1	P40	+	+	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+
29	1	P42	+	+	+	-	+	+	+	+	-	+	+	-	+	+	-	-	+
30	1	P43	+	+	+	-	+	+	+	+	-	+	+	+	+	+	-	+	+
31	1	P44	+	+	+	-	+	-	+	+	-	+	+	+	+	+	-	+	+
32	1	P45	+	+	+	-	+	+	+	+	+	+	-	-	+	+	+	+	+
33	1	P46	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	-	+
34	1	P48	+	+	+	-	+	+	+	+	-	+	+	-	+	+	+	+	+

+: The bacteria are resist to that antibiotic.
-: The bacteria are sensitive to that antibiotic

Table 3: Number of transforment colonies of *E. coli* DH5α

Isolate	No. of transformant colonies	No. of colonies that grow on nutrient agar containing antibiotics at final concentration µg/ml																
		Amp	Te	Str	Tob	Rif	Nal	Nit	Tm	Cef	Cep	Ch	Cip	Ery	Gm	Lin	Pn	Pip
P1	99	3	6	*S	S	90	S	S	7	99	99	75	S	70	23	N	80	80
P8	36	6	10	10	**N	2	6	7	5	26	25	20	27	15	7	N	29	28
*E.coli*DH5α Competant cell		S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

*S: No growth of transformant colonies obtained
**N: The bacteria are sensitive to such antibiotics

one antibiotics group 5, 7, 11 and 20. Ten isolates 20% were sensitive to two antibiotics, while other 25 isolates 50% were sensitive to 3-5 antibiotics from seventeen used antibiotics.

To determine if the antibiotic resistance in isolated *P. aeruginosa* is encoded by plasmid DNA or chromosomal DNA transformation process was performed to the most resistant isolate of *P. aeruginosa* P8 and more sensitive isolate P1 and *E. coli* DH5α strain. Extracted plasmid DNA from *P. aeruginosa* transferred successfully to *E. coli* DH5α strain (Table 3). Ninety nine and thirty six transforment colonies obtained for P1 and P8 isolates, respectively, these colonies sub-cultured on different antibiotics agar plates, and less than these numbers obtained.

DISCUSSION

P. aeruginosa is an opportunistic pathogen that causes human infection; it can be isolated from different environments. Appeared high resistance to the most widely used antibiotics and some isolates (Table 1) to all antibiotics. All isolates except group (2), show resistance to more than five antibiotics, and this phenomenon is called multiple resistance (MDR). MDR pseudomonas has been previously reported (Loureiro *et al*., 2002; Mahboobi *et al*., 2006; Sabtcheva *et al*., 2003). In this study all fifty *P. aeruginosa* isolates were resist 100% to Amp, Cln, Cef, Er, Gm, Pn and Lin (Table 2). Ineffectiveness of Amp, Cln, Cef, and Gm against *P. aeruginosa* been reported by (Khider, 2006; Hamasalih and Khider, 2009; Seshadri and Chhatbar, 2009; Okonko *et al*., 2010).

Transformation process results (Table 3) derives us to conclude that all genes responsible for antibiotic resistant in *P. aeruginosa* P1 and P8 isolates except Lin in P1 and Lin and Tob genes in P8 isolate are not chromosomally coded, this increase the possibility distribution of resistance for Amp, Tet, Str, Rif, Nal, Nit, Tm, Cef, Cep, Ch, Cip, Ery, Gm, Pn, Pir among pathogenic bacteria by transformation, conjugation and transduction (Sabtcheva *et al*., 2003) than Lin and Tob genes which are chromosomally coded. Number of purified transformant colonies produced by the plasmid DNA of tested isolates were less than that which started from, such for P1, the number of transformant colonies obtained were 99 and 36 colonies for P1 and P8 respectively, less numbers obtained after subcultured on agar plates containing tested antibiotics separately, this mean that the antibiotic resistant genes are located on different fragments of R-plasmid (Snyder and Champness, 1997), one of these which called RTF (resistance transfer factor), as it is comprises all the genes necessary for conjugation and is of large size. Other fragment which is called the r-determinant, it contains all the genes confer antibiotic resistant except the tetracycline-resistance

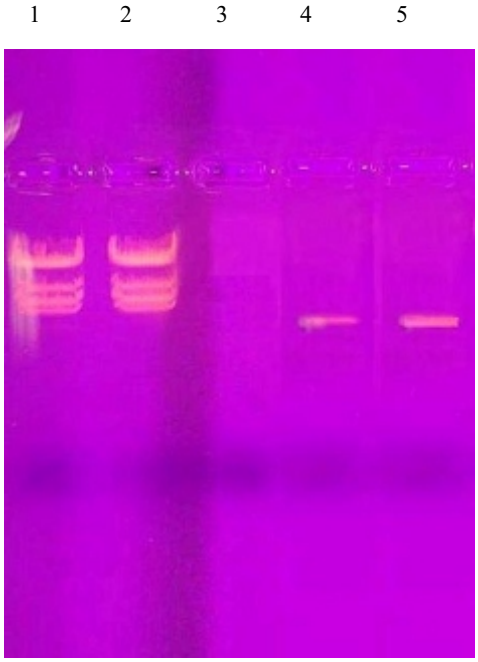

Fig. 1: The plasmid profile of *P. aeruginosa*
Lane1: P8 *P. aeruginosa* isolate
Lane 2: P1 *P. aeruginosa* isolate
Lane 3: *E. coli* DH5alfa strain
Lane 4: Plasmid from P1 transformed to *E. coli* DH5α
Lane 5: Plasmid from P8 transformed to *E. coli* DH5α

determinant which is on RTF. These two fragments of R-plasmid may enter the DH5α host in different efficiency because of their variation in size (Khider, 2002). In addition, irregular segregation of plasmid DNA species may result difference in the transformant colonies for other antibiotics. The transforment colonies fail to grow on nutrient agar supplemented with Tobramycine in P8 isolate, this finding may be has related to that the genes which responsible for resistance to this antibiotic either located on chromosomal DNA or on large plasmid which can not enter the DH5α strain acting as a host.

From the findings of this study, it is evident that there were relatively high incidences of MDR pseudomonas in Sulaymai city, and relatively low effects of the tested antibiotics on these MDR bacteria. This has been attributed to either the ability of these organisms to produce resistance mechanisms, for this the characterization of plasmid DNA for P1 and P8 *P. aeruginosa* isolates indicated that these two isolates transfer to another bacteria, in present study to *E. coli* DH5α strain (plasmid less strain Fig. 1 lane 3) lane 4 and 5 for P1 and P8 *P. aeruginosa* isolates respectively. More over the antibiotic resistance genes can be distributed naturally among bacteria through conjugation and transduction process (Gillespie and Hawkey, 2006), or

poor quality of antibiotics available in the city or lesser concentration of antibiotics incorporated, or antibiotics are availably to the public, these factors could enhanced the high incidence rate of resistant pathogenic bacteria in the Hospitals, however the antibiotics are often used in animals and the antibiotics sold over the counter without a prescription which compounds the problem, however misuse and overuse of antibiotics by doctors as well as patients should not be neglected. Therefore, our recommended that more restrictions on the irrational use of antibiotics and public awareness activities should be undertaken to alert the public to the risks of the unnecessary use of antibiotics.

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