Genetic Study of Multiple Antibiotic Resistances of *Pseudomonas aeruginosa* Isolated from Human Infections

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Abstract

Thirty-two isolates of *Pseudomonas aeruginosa* were isolated from different human infections (wound, burns, urine and ear) and identified on the bases of cultural, morphological and biochemical tests. All isolates showed resistance to Ampicillin, Amoxicillin, Carbencillin, Chloramphenicol, Cefotaxime**,** Erythromycin, Lincomycin, Penicillin, Tetracycline and Trimethoprime , while they appeared variable resistances to Amikacin**,** Ciprofloxacin, Gentamicin, Neomycin, Rifampicin and Streptomycin. All isolates produced pyocyanin on King's agar medium and sixteen of them produced extracellular proteases on skimmed milk agar plats.

DNA contents study of the isolates revealed that the presence two plasmid profiles on agarose gel electrophoresis: the non-proteases producing isolates showed the presence of one or two small plasmids , whereas the other profiles of protease producing isolates revealed that the presence large single plasmids in agarose gel electrophoresis.

To determine the location of antibiotic resistance genes of *P. aeruginosa* isolates ,the genetic transformation and bacterial conjugation experiment were performed by using the standard plasmidless *Escherichia coli* MM294 strain as a recipient and pseudomnal isolates as donor. The genetic transformation results appeared successful transfer of plasmid DNA contents after extracted from P4, P16, P22 and P27 isolates and transformed into *E.coli* MM294 at frequencies 26 x 10^{-2}, 56 x 10^{-2} , 49 x 10^{-2} and 42 x 10^{-2} respectively. The transformed cells acquired multiresistance to most of the studied antibiotics (ampicillin, amikacin, amoxicillin, carbencillin, chloramphenicol, cefotaxime, erythromycin, penicillin, streptomycin, tetracycline, and trimethoprim). The results indicated that the plasmid carrying multiple resistance genes encoding at least 7 to11 antibiotic resistances in transformed cells and *P. aeruginosa* isolates, while the antibiotic resistance genes of Lincomycin are encoded by chromosomal DNA. Also found all of the transformant colonies are non producing to pyocyanin pigment .The mobilization ability of plasmids were studied, they appeared that the plasmids of P9, P15, P22, and P26 isolates were successfully transferred into *E. coli* MM294 at frequencies 88x10^{-2}, 455x10^{-3}, 122x10^{-2}, and 154x10^{-2} respectively, the transconjugants acquired resistance to most antibiotics excerpt erythromycin, carbencillin and Lincomycin resistance gene that may be due to the location of their resistance genes either on the non-transmissible plasmid or on the chromosomal DNA of *P. aeruginosa* isolates. Whereas the plasmid of *P. aeruginosa* P18 is appeared non-conjugative plasmid . All tested transconjugant colonies produced pyocyanin pigment on King's agar medium supplemented with glucose but they had not ability to produce extracellular proteases on skimmed milk agar plats which indicated that the genes encoding proteases production are encoded chromosomally.

From this study was concluded that the transfer of multiple drug resistances between enterobacteria and *P. aeruginosa* may take place in nature. The production of pyocyanin in the transconjugant colonies could be related to the presence of the plasmid R68.45 carrying the genes encoding Amp, Gm and Tc resistances. This plasmid is available in the *P. aeruginosa* isolates which can be integrated with chromosomal DNA at adjacent position of the genes encoding pigment production that may be transmissible by plasmid after that transferred into the *E.coli* MM294 strain

دراسة وراثية للمقاومة المتعددة للمضادات الحيوية في بكتريا *Pseudomonas aeruginosa* المعزولة من اخماج مختلفة في الانسان

الخلاصة

عزلت اثنا وثلاثون عزلة من بكتريا *Pseudomonas aeruginosa* من أخماج مختلفة في الإنسان التي شملت أخماج الجروح والحروق والاذن وأخماج الجهاز البولي. تم تشخيص العزلات بالاعتماد على الخصائص المجهرية والزرعية والكيموحيوية.

تم دراسة بعض عوامل الضراوة مثل الحساسية للمضادات الحيوية وإنتاج صبغة البايوسيانين (Pyocyanin) والبروتييزات الخارجية. أظهرت النتائج بأن كافة العزلات مقاومة للامبسلين والاموكسيسيلين والكاربنسلين والكلورامفينكول والسيفاتكسيم والارثرومايسين واللنكومايسين والبنسلين والتتراسايكلين والترامثبرم، في حين أنها أظهرت تبايناً في مقاومة الاميكاسين والسبروفلاكساسين والجنتامايسين والنيومايسين والريفامبسين والستربتومايسين. وكانت كافة العزلات منتجة لصبغة البيوسيانين في وسط King's agar بينما كانت ستة عشر عزلة منتجة للبروتييزات الخارجية على وسط حليب الفرز.

اظهرت نتائج دراسة النسق البلازميدي في هلام الاكاروز بأن العزلات غير المنتجة للبروتييز تمتلك بلازميدات صغيرة الحجم فقط. في حين ان العزلات المنتجة للبروتييز تمتلك بلازميد كبير مفرد مشترك و بلازميدات صغيرة الحجم. ولتحديد موقع جينات المقاومة سواء كانت بلازميدية او كروموسومية تم اجراء تجارب التحول الوراثي والاقتران بين عزلات البكتريا كسلالات واهبة والبكتريا القياسية الخالية من البلازميدات (*E. coli* MM294(rif' كسلالة مستلمة للبلازميدات. تم نقل الدنا البلازميدي المستخلص من السلالات الواهبة *P. aeruginosa* P4, P16, P22 and P27 بالتحول الوراثي إلى الخلايا المؤهلة لبكتريا *E. coli* MM294(rif') الخالية من البلازميدات بتردد قد بلغ 26×10^{-2} و 56×10^{-2} و49×10^{-2} و 42×10^{-2} على التوالي. واظهرت النتائج بان كافة الخلايا المتحولة قد اكتسبت المقاومة المتعددة للمضادات الحيوية باستثناء المضادات الارثروميسين والكانامايسين واللنكوميسين مما يشير الى ان هذه البلازميدات تشفر للمقاومة المتعددة للمضادات الحيوية في حين ان جين المقاومة للنكوميسين قد يكون محمولا على الكروموسوم. وقد وجد ان كل المستعمرات المتحولة غير منتجة لصبغة البايوسيانين.

تم دراسة قابلية البلازميدات على الانتقال بوساطة الاقتران البكتيري, وقد اظهرت العزلات P9 و P15 و P22 وP26 قابلية على الاقتران مع البكتريا القياسية الخالية من البلازميدات *E .coli* MM294 بانتقال البلازميدات اليها بتردد قد بلغ $88x10^{-2}$ و $455x10^{-3}$ و $122x10^{-2}$ و $154x10^{-2}$ على التوالي. اكتسبت الخلايا المقترنة المقاومة لمعظم للمضادات الحيوية باستثناء الارثرومايسين والكاربنسلين واللنكوميسين مما قد يشير الى ان الجينات المشفرة لهذه المقاومة قد تكون محمولة على بلازميدات غير مقترنة او على الكروموسوم. في حين اظهر البلازميد في العزلة *P. aeruginosa P18* بأنه غير اقتراني . بينت كافة الخلايا المقترنة المدروسة قابلية في انتاج صبغة البايوسيانين في وسط King المزود بالكلوكوز وغير منتجة لانزيمات البروتييز الخارجية في وسط الحليب الفرز مما قد يشير الى ان الجينات المشفرة للبروتييز ات هي جينات كروموسومية.

نستنتج من هذه الدراسة بان لبكتريا *P. aeruginosa* قابلية في الاقتران مع افراد العائلة المعوية في الطبيعة ونتيجة لذلك تنتشر المقاومة المتعددة للادوية . إن إنتاج صبغة البايوسيانين في الخلايا المقترنة قد يشير إلى وجود البلازميد R68.45 الذي يشفر لمقاومة الامبسلين والجنتاميسين والتتراسايكليين , ولهذا البلازميد قابلية على الانغراس او الاندماج مع الكروموسوم البكتيري وقد يكون هذا الموضع قريب من الجينات المشفرة

للبايوسيانين ونتيجة لذلك انتقلت الى البلازميد الذي قام بدوره بنقلها إلى الخلايا المقترنة من بكتريا *E.coli* MM294.

Key word: *P. aeruginosa*, plasmid profile, antibiotic resistance, pyocyanin -
بحث مستل من أطروحة الماجستير للباحث الثاني.

Introduction

P. aeruginosa is an opportunistic pathogen of human, belonging to the bacterial family Pseudomonadaceae, that is widespread in the environment, a major cause of community acquired infections.They are Gram negative, aerobic, motile and rod-shaped bacteria, measuring about (0.5 to 0.8 µm by 1.5 to 3.0 µm), and occur as single bacteria, in pairs and occasionally in short chains (Todar, 2004). It grows well at 37 - 42°C and they are oxidase positive; many strains produce two types of soluble pigments, the fluorescent pigment (pyoverdin) and the bluish pigment (pyocyanin). Identification is usually based on colonial morphology, oxidase positivity, and presence of characteristic pigments and growth at 42°C (Jawetz *et al*., 1998).

P. aeruginosa is a bacterium responsible for severe nosocomial infections, life-threatening infections in immunocompromised persons, and chronic infection in cystic fibrosis patients (Delden and Iglewski, 1998). In hospital, the bacterium is the leading cause of nosocomial lung infections and a common cause of wound infections, especially of thermal burns. *P. aeruginosa* is responsible for 16% of nosocomial pneumonia cases, 12% of hospital acquired urinary tract infections, 8% of surgical wound infections, and 10% of blood stream infections (Pollack, 1995). *P. aeruginosa* is notorious for its resistance to antibiotics, and is, therefore, particularly dangerous and dreaded pathogens. The bacterium is naturally resistant to many antibiotics due to the permeability barriers afforded by its outer membrane lipopoly saccharide (LPS). Also, its tendency to colonize surfaces in a biofilm form makes the cells impervious to therapeutic concentration antibiotics (Baron and Finegold,1990).

Moreover, *Pseudomonas* maintains antibiotic resistance plasmids, both R-factor and resistance transfer factors (RTF), and it is able to transfer these genes (Todar, 2004). Most strains of *P. aeruginosa* are multidrug resistant, and contain R-plasmid especially clinical strains with different molecular weight (1.91– 40) Mega Dalton. Moreover, it contains high molecular weight plasmids (60 – 100) Mega Dalton ((Tsakris *et al*., 1992).

The bacterium's virulence depends on a large number of cell-associated and extracellular factors; certain strains release an extracellular slime that is lethal to mice and protects the pathogen from phagocytosis, and the slime is a virulence factor for *P. aeruginosa* (Delden and Iglewski, 1998). On the other hand, the study of nosocomial bacteria like *P. aeruginosa* at the molecular genetics level is important, since this bacteria has the ability to transfer its genetic materials specially antibiotic resistant genes to another bacteria through conjugation and also transformation processes which become a great problem in chemotherapy. Controlling of antibiotic resistance gene at the molecular levels such as by using curing agents with appropriate concentration may limit or release the resistance of pathogenic bacteria (Hardy, 1986). Most strains of *P. aeruginosa* produce two exotoxins, exotoxin A and exo-enzyme S,

and a variety of cytotoxic substances including phospholipases, pyocyanin, rhamnolipids and proteases; an alginate-like exopolysaccharide is responsible for the mucoid phenotype. The importance of these putative virulence factors depends upon the site and nature of infection. Proteases play a key role in corneal ulceration, and are important in burn infection; and associated with chronic pulmonary colonization (Todar, 2004). This research deals with spread multiple resistance of antibiotics between *P. aeruginosa* isolated from different infections in human and related this with plasmid profile .Also study the ability of plasmid to transfer and spread of .antibiotic resistances

Materials and Methods

Isolation and identification of *P.aeruginosa* .1

The samples collected from different human infections were transferred to the laboratory and activated using brain heart infusion broth. After activation inoculated on the MacConKey agar, a single colonies were selected, for more purification inoculated on the selective medium cetrimide agar, and oxidase test was done, positive isolates, and microscopically Gram negative rod shape, identified provisionally as *P.aeruginosa*, subcultured on nutrient agar slants, after incubation at 37 °C for 24 hr., .((stored at 4 °C, till other bacteriological tests were done (Holt *et al*., 1994
The api 20E Micro tube system (BioMerieux SA, Lyon, France) was used. This system is a standardized, miniaturized version of conventional procedures for the .identification of Enterobacteriaceae and other Gram negative bacteria

Antibiotic susceptibility test .2

Antibiotic susceptibility test by disk-diffusion method was performed according to Bauer et al. method (1966) that described in (Baron and Finegold,1990) and the .(results was compared with standard inhibition zone according to Wikler *et al*. (2006
Antibiotic resistance test by pour method was used to screen the genetic transfer of antibiotic resistances in studied isolates was preformed according to Sambrook *et* .(*al*. (1989) and Baron & Finegold (1990

Total DNA extraction by salting out method .3

Total DNA content of *P. aeruginosa* isolates was extracted according to salting out method (Pospiech and Neuman, 1995). Plasmid DNA content was extracted using .(alkaline lyses (Sambrook *et al*., 1989

Agarose Gel electrophoresis technique .4

Agarose Gel was prepared according to the method of Sambrook *et al*. (1989); agarose gel was prepared using 0.7% agarose gel. The electrophoresis were run at

6volt/cm for 3hrs. The gels were illuminated with ultraviolet transilluminator , and then photographed by digital camera.

Bacterial Conjugation .5

The transmissible ability of DNA plasmid of *P. aeruginosa* was tested according to Olsen *et al*. (1992) method by *P. aeruginosa* isolates as donor and standard plasmidless strain of *E .coli* MM294 Rif^r as recipient. The conjugation frequency was calculated according to the following equation:

$$\text{The frequency of conjugation} = \frac{\text{No. of transconjugant cells / ml}}{\text{Total No. of recipient cells / ml}}$$

Genetic transformation .6

The genetic transformation was performed according to the Sambrook *et al*.(1989) using free plasmid that extracted from *P. aeruginosa* isolates (donors) and standard plasmidless strain of *E .coli* MM294 Rif^r as recipient. The genetic transformation frequency was calculated according to the following equation:

$$\text{The transformation frequency} = \frac{\text{No. of transforments (cells / ml) / DNA amount (µg}}{\text{Total number of competent cells / ml}}$$

Results and Discussion

Isolation and identification of *Pseudomonas aeruginosa* .1

A total of ninety four samples were collected from different human infections (ear, urine, wounds, and burns), from the General, Urology, Teaching, and Emergency Hospitals in Sulaimaniya City.

All bacterial isolates were characterized selectively using cetrimide medium, cultural and morphological characteristics but only thirty two isolates were indicated as *P. aeruginosa*. The colonies of *P. aeruginosa* isolates were studied using nutrient agar plates and MacConkey's agar plates. They are small in size, fried-egg appearance, smooth with flat edge and an elevated appearance, while the others which are isolated from the secretions of urinary tract infections have a mucoid appearance on nutrient agar. The smooth and mucoid colonies are presumed to play a role in colonization and virulence.

All of these isolates produce pyocyanin (blue green pigment), which is in accordance with that is mentioned by Todar (2004). *P. aeruginosa* does not ferment lactose and is differentiated from lactose fermenting bacteria (Enterobacteriaceae). Culture is the specific test for diagnosis of *P. aeruginosa* infection. The bacterial cells from smear preparation are gram negative, rod-shaped, and occur as single, in pairs, or in short chains, presumptively regards *P. aeruginosa,* which in accordance with previous observation (Jawetz *et al*., 1998; and Todar, 2004).

The bacterial colonies are able to grow at 41°C but not at 4°C; These criteria are used for the identification of *P. aeruginosa* from other species; this is agreement with Jawetz *et al*. (1998), who found that *P aeruginosa* have the ability to grow at 41°C

and produce pyocyanin after growing on cetrimide medium. Sixteen of *P. aeruginosa* isolates produced extracellular proteases. Furthermore, biochemical tests were performed to support the results above, using api 20E test which is the rapid accurate technique for the identification of the family Enterobacteriaceae and Gram negative .(bacilli (Kurlandsky and Fader, 2000

All the isolates are oxidase positive, which is regarded an important characteristics for these bacteria as mentioned by (Bingen *et al*., 1992); identification of *P. aeruginosa* strains is usually based on clinical morphology, oxidase positively, the presence of characteristics pigments, and growth at 42C as described by (Jawetz *et al*., 2001). In general, according to the analytical profile index, (1997), which is (98%) indicate that all isolates were *P. aeruginosa*. The bacterial isolate takes the letter (P) .(and the number of samples that isolated from (1 to 32

The results showed that the urine isolates were the most frequent abundance that encountered 13, while for burns; wounds and ear were 9, 7, and 3 respectively. This may be due to the fact that these samples were not taken regularly, but can therefore be considered as reflections of the actual situation of *Pseudomonas aeruginosa* of the .(patients in these hospitals (Table 1

Table (1): *P. aeruginosa distribution* according to source of isolation

Source of isolation	.Isolate No	No. of isolate	of % isolate
Urine	4,5,6,7,8,9,10,11,12,13,14,15,16	13	40.6
Burns	22,24,25,27,28,29,30,31,32	9	28.1
Wound	17,18,19,20,21,23,26	7	21.9
Ear	1,2,3	3	9.4

In Tunisian, Pallilo and Salleh (1992) found that the percent of *P. aeruginosa* was 65% for respiratory tract, 13.5% for urine, 8.5% for wounds and 13% for others .among 213 isolates

In another study in India, Puri *et al.* (1996) obtained 30% from wound, 30.5% from urine and 15.2% from stool specimens. Tassion *et al.* (1998) indicated 30% for urine, 14% in pus, 9% in sputum and wound among 88 isolates in 11 Greek hospitals. Also Delden and Iglwesky (1998) reported that *P. aeruginosa* is responsible for 16% of nosocomial pneumonia cases, 12% of hospital acquired urinary tract infections, 8% of surgical wound infections, and 10% of blood stream infections. Although there are differences in the percent of infection between our results and others, these results still agree with that which says *P. aeruginosa* is an opportunistic pathogen that causes human infections and can be isolated from soil, water and disinfectants (Marques *et al.*, 1979; Tassois *et al*., 1998; Abd Al-Amir, 1999 and Todar, 2004).

Antibiotic resistance of *P. aeruginosa* isolates .2

P. aeruginosa is currently one of the most frequent nosocomial pathogen and the infection is often difficult to treat due to multiple resistance of antibiotic (Emori and Gayner, 1993). It is a common phenomenon in most general hospitals that the frequency of occurrence of infections caused by *P. aeruginosa* is increasing. The reasons for the increase are not completely understood, but undoubtedly they bear some relationship to the widespread use of antibiotic therapy and the resistance of *P. aeruginosa* to most of the widely used antibiotics. It is not unusual for strains of this organism isolated from infections to be resistant to three or more of the following antibiotics: sulphadiazine, ampicillin, kanamycin, streptomycin, chloramphenicol, neomycin, or tetracycline. The only antibiotic to which *P. aeruginosa* is usually .sensitive is polymyxin, although strains resistant to this agent are common

The mechanism of resistance to antibiotics includes, reduced cell wall permeability, production of chromosomal and plasmid mediated ß-lactamase (Livermore, 1989), aminoglucoside-modifying enzymes (Prince, 1989), and an active .(multi drug efflux mechanism (Li *et al*., 1994

Thirty two (32) *P. aeruginosa* isolates were screened for their resistance to sixteen widely used antibiotics in medicine which are ampicillin, amikacin, amoxicillin, carbencillin, ciprofloxacin, chloramphenicol, cefotaxime, erythromycin, gentamycin, lincomycin, neomycin, penicillin, rifampicin, streptomycin, tetracycline, and trimethoprim (Table 2). All isolates show resistance to ampicillin, amoxicillin, carbencillin, chloramphenicol, cefotaxime, erythromycin, lincomycin, penicillin, tetracycline and trimethoprim), while they show variable resistance to amikacin, ciprofloxacin, gentamycin, neomycin, rifampicin and streptomycin. Table (2) revealed that *P. aeruginosa* isolates revealed high resistance to most widely used antibiotics in medical treatment, results in an increased frequently of resistance in microbial flora and *P. aeruginosa*. The reasons for this pattern of resistance to such a wide variety of drugs are not understood at the biochemical level. This also could be a .fruitful avenue of future research, with both academic and practical implications

In recent years it has been emphasized that there is a remarkable increase in the incidence of infection by antibiotic resistance microorganisms in different parts of the world, for example Flick and Cluff (1976) found that *P. aeruginosa* isolate from patients demonstrated resistance to carbencillin, while Marques *etal.* (1979) observed that some of *P. aeruginosa* isolates from soil and water resisted gentamycin, where as all isolates resisted ampicillin, chloramphenicol, nalidixic acid, tetracycline and .streptomycin

The epidemiology of drug resistance in the Enterobacteriaceae and some of the gram-positive cocci undergo a remarkable change in character with the widespread occurrence of resistance transfer factors (RTF). RTF may transfer to drug-sensitive strains by conjugation in much the same way and with much the same type of kinetics as F transfer in *E. coli*. Furthermore, RTF can act as sex factors in promoting conjugation and transfer of chromosome Small *et al*. (1993). Jiban (1986) found that among (83) *P. aeruginosa* isolates 29% were resistant to carbencillin, while all isolates were resistant to ampicillin, cefotaxime, chloramphenicol, tetracycline, trimethoprim, and streptomycin. These results were relatively the same as results achieved in our

study, and we can say that our results were in agreement with Jiban (1986). Tassior *et al*. (1998) obtained that 10% out of 88 isolates of *P. aeruginosa* were resistant to all antibiotics used. Quadri *et al.* (1994) reported that the pattern of antibiotic resistance of bacterial pathogen usually varied from one geographic location to another and outbreak of disease caused by multiple resistant bacteria had occurs more frequently in developing countries.

Antibiotic resistance is now generally accepted as a major public health issue, and these problem should be solved. Shahid (2004) reported that antimicrobial susceptibility of the multi drug resistant *P. aeruginosa* isolates were for AK 100%, Tb. 80%, Gt 30%, Nt. 70%, Cr. 40%, Cp. 20% C2. 20%, and 40%, 30%, 80%, for Ct., Ce, Cl. respectively.

The fluorinated quinolones, in particular ciprofloxacin, are still active against *P. aeruginosa*. Resistance may nevertheless, emerge during long term treatment of chronic infections. Resistance to other antibiotic including cephalosporin's and antipseudomonal antibiotics may also occur in future (Shahid, 2004). Given this drug-resistant nature of *P. aeruginosa*, it is important from a public health viewpoint to know whether RTF can either occur in this species or be transferred to it from the enterobacteria.

Table (2): The resistance of *P. aeruginosa* isolates to antibiotics

Antibiotic Sensitivity Tests

No. of *P. aeruginosa* Isolates	Source of Isolation	Ap	AK	Ax	Car	Cip	Cm	CTX	Ery	Gm	Lin	N	Pi	Rif	Sm	Tc	Tri
1	Ear	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
2	Ear	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
3	Ear	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
4	Urine	+	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+
5	Urine	+	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+
6	Urine	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
7	Urine	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
8	Urine	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
9	Urine	+	+	+	+	-	+	+	+	-	+	-	+	-	+	+	+
10	Urine	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
11	Urine	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
12	Urine	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
13	Urine	+	+	+	+	-	+	+	+	-	+	-	+	-	+	+	+
14	Urine	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
15	Urine	+	+	+	+	-	+	+	+	-	+	-	+	-	+	+	+
16	Urine	+	+	+	+	-	+	+	+	-	+	-	+	-	+	+	+
17	Wound	+	+	+	+	-	+	+	+	(I)	+	+	+	+	+	+	+

Antibiotic Sensitivity Tests

No. of *P. aeruginosa* Isolates	Source of Isolation	Ap	AK	Ax	Car	Cip	Cm	CTX	Ery	Gm	Lin	N	Pi	Rif	Sm	Tc	Tri
18	**Wound**	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+
19	**Wound**	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
20	**Wound**	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
21	**Wound**	+	**(I)**	+	+	-	+	+	+	-	+	+	+	+	+	+	+
22	**Burn**	+	+	+	+	-	+	+	+	-	+	-	+	-	+	+	+
23	**Wound**	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
24	**Burn**	+	-	+	+	-	+	+	+	+	+	-	+	+	+	+	+
25	**Burn**	+	+	+	+	**(I)**	+	+	+	+	+	+	+	**(I)**	+	+	+
26	**Wound**	+	+	+	+	**(I)**	+	+	+	+	+	+	+	-	+	+	+
27	**Burn**	+	-	+	+	-	+	+	+	-	+	-	+	-	-	+	+
28	**Burn**	+	+	+	+	**(I)**	+	+	+	+	+	+	+	-	+	+	+
29	**Burn**	+	**(I)**	+	+	+	+	+	+	+	+	-	+	+	-	+	+
30	**Burn**	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+
31	**Burn**	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
32	**Burn**	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+

The symbols (+): Resistance to Antibiotics, (-): Sensitive to Antibiotics, and (I): * .intermediate

Ap: ampicillin, Ak: amikacin, Ax: amoxicillin, Car: carbencillin, Cip: * * ciprofloxacin, Cm: chloramphenicol, Ctx: cefotaxime, Ery: erythromycin, Gm: gentamycin, Lin: lincomycin, N: neomycin, Pi: penicillin, Rif: rifampicin, Sm: .streptomycin, Tc: tetracycline, and tri: trimethoprim

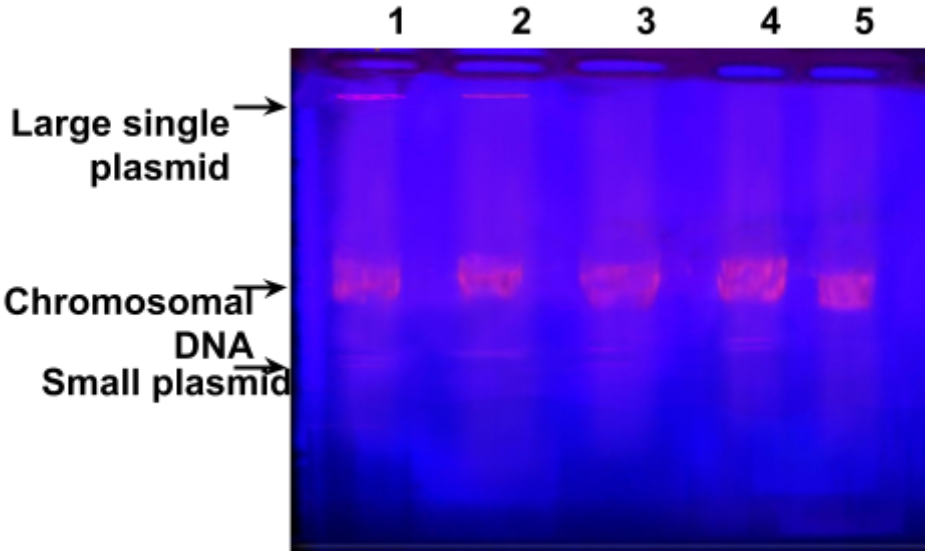
Therefore, to combat this problem, effort showed be made to isolate and characteristic plasmids responsible for resistance in multi drug resistance (MDR) *P. aeruginosa* strain from all over country and a nation wide antibiotic policy should be defined after evaluating the effectiveness of the regime so that the misuse of antibiotics is minimized and also the emergency of multi drug resistant organism can be restricted. This is a preliminary study on plasmid mediated 16 antibiotic resistance in *P. aeruginosa* isolates; however, there is a need for a large scale study to find out the plasmid mediated drug resistance in *P. aeruginosa* along with isolation with .isolation and characterization of plasmids

The plasmid profile of *P. aeruginosa* isolates .3

Electrophoresis characterization of total DNA and plasmid DNA content of *P. aeruginosa* isolates obtained from different human infections were extracted by salting out and alkaline lysis according to Kado and Liu (1981) respectively and .carried out for migration using 0.7 % agarose gel, at 50 volt for 13 hours

Figure (1) shows the plasmid profile of four Pseudomonal isolates which represent two groups, the first one non producer to extracellular protease (P12 and p16 isolates) and the second group had the ability to produce extracellular protease (P22 and P24 isolates). The results revealed two plasmid profiles: one of them indicated the presence of one small plasmids (P24 and P12 isolates) and two small plasmids (P22 and P16 isolates), while the other plasmid profiles revealed large single plasmids (P22 .and P24 isolates) in agarose gel electrophoresis

The previous studies by Gabisoniia *et al*. (1992) and Tsakris *et al.* (1992) elucidated that plasmid size bearing antibiotic resistance characteristics in *P. aeruginosa* ranged between (20-100) mega Dalton. Nordmann (1993) found that the size of plasmid ranged between (1.9-45.0) MD also reported that the size of plasmid in the bacteria ranged between (4-80) Kbp. It is demonstrated from this study that the high resistance of the tested isolates to antibiotics may be related to the large size plasmids containing to the isolated carrying groups of antibiotic resistance genes (May .(be more than 11



.Fig. (1): The plasmid profile of *Pseudomonas aeruginosa* isolates

The DNA plasmid extracted by alkaline lyses (Kado and Liu, 1981) and migrated .on agarose gel 0.7%, 50 volt, for 13hr

.Lane 1: DNA content of proteases producing *P. aeruginosa* P22 isolate

.Lane 2: DNA content of proteases producing *P. aeruginosa* P24 isolate

.Lane 3: DNA content of non-proteases producing *P. aeruginosa* P12 isolate

.Lane 4: DNA content of non-proteases producing *P. aeruginosa* P16 isolate

.Lane 5: DNA content of standard plasmidless strain *E.coli* MM294

Genetic transfer of *P. aerurginosa* plasmids by transformation .4

The transformation experiments were carried out according to Davis *et al.* (1986) by using *P. aeruginosa* strain as the donor (P4, P16, P22, and P27 isolates) and the plasmidless competent cells of *Escherichia coli* MM 294 (Rif^r) as the recipient strain. The transformation results revealed that the transformed *E.coli* P4 cells acquired multiresistance to ampicillin, amikacin, amoxicillin, carbencillin, chloramphenicol, cefotaxime, erythromycin, penicillin, streptomycin, tetracycline, and trimethoprim, while they were sensitive to each of ciprofloxacin, gentamycin, and lincomycin. That result indicated that the plasmid carrying multiple resistance genes encoding 11 antibiotic resistances in transformed cells and *P. aeruginosa* P4, while the antibiotic resistance genes of Lincomycin are encoded by chromosomal DNA and this agrees .(with that result obtained by(Quadri *et al.*, 1994

Table (3): The transformation frequency and number of transformed .colonies among four strains of *P. aeruginosa* isolates

P. aeruginosa and Transformed strains	(Antibiotics resistance (µg/ml															Frequency
	Ap	Ak	Ax	Car	Cip	Cm	Ctx	Ery	Gm	Lin	Pi	Rif	Sm	Tc	Tri	
P. aeruginosa P4	+	+	+	+	-	+	+	+	-	+	+	-	+	+	+	
E. coli P4	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	x 26 10^{-2}
P. aeruginosa P16	+	+	+	+	-	+	+	+	-	+	+	-	+	+	+	
E. coli P16	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+	x 56 10^{-2}

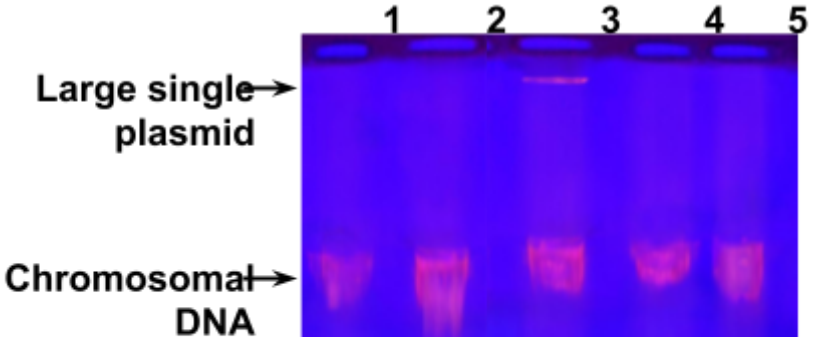
P. aerugino sa P22	+	+	+	+	-	+	+	+	-	+	+	-	+	+	+	
E. coli P22	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	x 49 10^{-2}
P. aerugino sa P27	+	-	+	+	-	+	+	+	-	+	+	-	-	+	+	
E. coli P27	+	-	+	-	-	+	+	+	-	-	+	+	-	-	-	x 42 10^{-2}
E. coli MM294 control) recipient (	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	

*The *E. coli* MM294 Rif^r recipient concentration was (2×10^6) cell/ml.

* The symbols (+): Resistance to Antibiotics, (-): Sensitive to Antibiotics.

Ap: ampicillin, Ak: amikacin, Ax: amoxicillin, Car: carbencillin, Cip: ciprofloxacin, Cm: chloramphenicol, Ctx: cefotaxime, Ery: erythromycin, Gm: gentamycin, Lin: lincomycin, Pi: penicillin, Rif: rifampicin, Sm: streptomycin, Tc: tetracycline, and Tri: trimethoprim.

The transformed *E.coli* P16 cells acquired multiresistance to ampicillin, amikacin, amoxicillin, penicillin, streptomycin, tetracycline, and trimethoprim, while they were sensitive to each of carbencillin, ciprofloxacin, chloramphenicol, cefotaxime, erythromycin, gentamycin and lincomycin. These results speculated that the plasmid carrying multiple resistance genes encoding 7 antibiotic resistances in transformed cells and *P. aeruginosa* P16, while the sensitivity to that 8 antibiotics may be refer to that the antibiotic resistance genes located on large plasmid or chromosomal DNA rather than small plasmid.



.Fig. (2): The plasmid profile of the transformed cells of *E. coli* MM294

The DNA plasmid extracted by alkaline lyses (Kado and Liu, 1981) and migrated .on agarose gel 0.7 %, 50 volt, for 6hr

.Lane 1: DNA content of *P. aeruginosa* P22 isolate, which have one small plasmid

.Lane 2: DNA content of transformed *E. coli* P22 isolate that contain small plasmids

Lane 3: DNA content of *P. aeruginosa* P16 isolate, which have two small plasmids .with single large plasmid

Lane 4: DNA content of transformed *E. coli* P16 isolate that contains two small .plasmids

.Lane 5: DNA content of standard plasmidless strain *E .coli* MM294

The transformed *E. coli* P22 cells acquired multiresistance to each of ampicillin, amikacin, amoxicillin, carbencillin, chloramphenicol, cefotaxime, erythromycin, penicillin, streptomycin, tetracycline, and trimethoprim while they were sensitive to each of ciprofloxacin, gentamycin, rifampicin, and lincomycin. These result indicated that the plasmid carrying multiple resistance genes encoding 11 antibiotic resistances in transformed cells as shown in Figure (2), while the sensitivity to lincomycin means that the responsible gene of Lincomycin encoded by chromosomal DNA and this .(agree with that obtained by (Quadri *et al.*, 1994

The transformed *E. coli* P27 cells acquired multiresistance to ampicillin, amoxicillin, chloramphenicol, cefotaxime, erythromycin and penicillin,while they were sensitive to amikacin, carbencillin, ciprofloxacin, lincomycin, streptomycin, tetracycline, and trimethoprim. These results indicated that the plasmid carrying the multiresistance genes which encoded 7 antibiotic resistances were transferred successfully to *E.coli* P27, while the other genes may be encoded by large single .plasmid or chromosomal DNA

Table (3) shows also that there are differences among isolates regarding the transformation frequencies, the highest recording for *P. aeruginosa* P16 (56×10^{-2}), P27 transferred at low frequency (42×10^{-2}), and (255×10^{-3}), (49×10^{-2}) were recorded for P4 and P22 respectively. These plasmids may be due to the size of transferred plasmid; small circle plasmids are transferred much more efficiently than .(large circle (Hardy, 1986

The plasmid DNA of *P. aeruginosa* P15 was not successfully transferred even after repeating the transformation process several times. The large size of the plasmid

may have been exposed to breakage during their preparation. This could be considered as a reason for the failure of transformation of those isolates (Hardy, 1986). We can conclude that the laboratory *E. coli* MM 294 strain treated with $CaCl_2$ can represent an efficient host for a commendation of the plasmid DNA transfer of *P. aeruginosa* .((Quadri *et al.*, 1994

Transformed MM 294 colonies were screened for pyocyanin production (the main characteristics of this species). After incubation for several days at 37°C, no pigment was observed to produce. This demonstrated that the genes responsible for pigmentation in *P. aeruginosa* are located on chromosome, and this agrees with .(previous reports of (Laird *et al*., 1980; and Bindereif and Neil ands, 1983

Most experimental work is necessary to confirm these results, for example curing in order to indicate that the genes responsible for production characteristics will remain after the curing process, also conjugation and cloning of the genes encoding .this pigment

The conjugative ability of plasmid DNA in *P. aerurginosa* isolates .5

The conjugation process was done in order to study the plasmid content profile of *P. aeruginosa*, and to find whether the plasmids encoded of drug resistance is conjugative or non-conjugative, up on mating of *P. aeruginosa* (P9, P15, P18, P22, and P26 isolates) with the *E .coli* MM294 the frequency of transconjugant colonies were $88x10^{-2}$, $455x10^{-3}$, $122x10^{-2}$, and $154x10^{-2}$ respectively selected on the selection media. The isolates of *P.aeruginosa* used were sensitive to rifampicin, and resistant to .ampicillin and streptomycin which are used as a genetic marker

All attempts failed to select transconjugant up on mating of *P. aeruginosa* P18 isolate with *E.coli* MM294, no transconjugant colonies were obtained on Muller Hinton agar plates containing ampicillin, streptomycin, and rifampicin; that means the plasmid contents of *P. aeruginosa* P18 isolate is non conjugative plasmid. Failure of obtaining transconjugant colonies may be due to their lack to one of the conjugation requirements in the donor strain (*mob* genes, *bom* sequence and formation of conjugation bridge). These results were confirmed by (Sagi *et al*., 1975), who found that among eleven clinical isolates of *P. aeruginosa* the plasmid contents of three of them were un-transmissible to *E. coli* .Also Pallilo and Salleh (1992) demonstrated that the efficiency of transfer was far lower than in similar types of transfer by *E. coil* strains possessing RTF. No attempts were made in this study to transfer the supposed .Pseudomonas RTF to sensitive strains of *P. aeruginosa*

The results shown in Table (4) indicate that mating has occurred between *P. aeruginosa* P9, P15, P22, and P26 isolates and *E. coli* MM294 and the frequency of transconjugant colonies obtained was $88x10^{-2}$, $455x10^{-3}$, $122x10^{-2}$, and $154x10^{-2}$ respectively. The growth of transconjugant colonies was tested on a Muller hinton agar containing carbencillin, chloramphenicol, erythromycin, gentamycin, lincomycin, tetracycline, and trimethoprim separately. The transconjugant colonies of *P. aeruginosa* P22 and P26 isolates appeared resist to all studied antibiotics except they are sensitive to carbencillin and erythromycin that may be due to the location of the antibiotic resistance genes either on the non-transmissible plasmid or on the .chromosome of *P. aeruginosa* isolates

From these studies, it is concluded that the transfer of drug resistance between enterobacteria and *P. aeruginosa* may take place in nature.

Chakrabarty, (1976) classified plasmids in *Pseudomonas* to P1, P2, P3 and other groups. Plasmid belonging to group (P1) can be transferred between varieties of gram negative including *E.coli*, while P2 and P3 are transmissible among *Pseudomonas* species but not *E.coli* constitute the P2 compatibility group.

On the other hand, all the transconjugant bacterial colonies obtained from a crosses with *E.coli* MM294 strain show resistance to the antibiotic lincomycin; the interpretation for this foundation involves that the *P. aeruginosa* isolates (act as a donor) contain plasmid called R68.45 (a derivative of R68). This plasmid is able to mobilize the bacterial chromosome from many origins (Haas and Holloway, 1976), and may be the Lin resistance gene included in this transfer. In addition, also all the transconjugant bacterial colonies obtained in this process show inability to produce the proteases, by those results we indicate that the genes responsible for proteases may be located on the chromosome or on the non-transmissible plasmids; this agrees with the results demonstrated by Michael *et al*. (1991), who observed the proteases gene encoded by chromosomal DNA.

Figure (3) shows the plasmid profile of transconjugant cells of *E.coli* MM294 acting as recipient through conjugation processes with *P. aeruginosa* (P9, P15, P22, and P26 isolates). These plasmid may be considered as self-transmissible and of different size, and they support the results above. Jacoby (1977) determined resistance to Amp, Gm, Sul, Tri and $HgCl_2$) which were non-conjugative in *P. aeruginosa* and found that a relatively small amount of chromosome are transferred, while Hardy (1986) elucidated that R100 plasmid in *E. coli* which conferred resistance to antibiotic including Tetracycline comprises all the genes necessary for conjugation.

.Table (4): Conjugation processes between *P. aeruginosa* and *E.coli* MM294

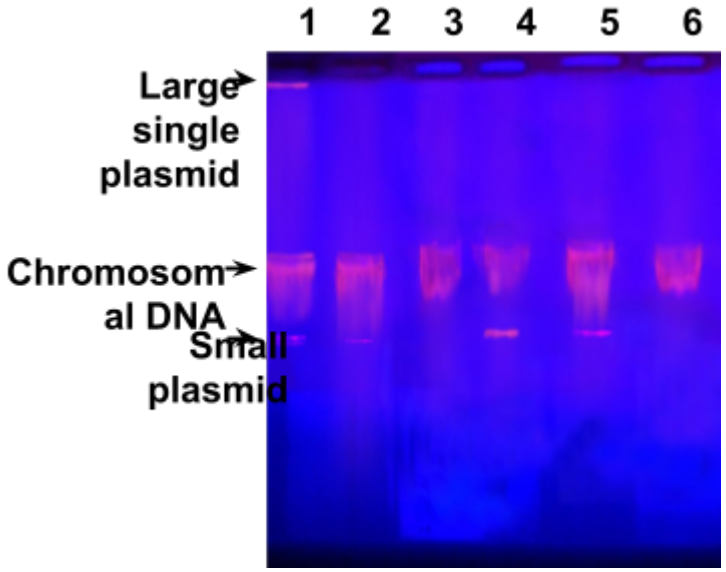
P.aeruginosa Donor	Transconjugant *E.coli* MM294	Antibiotic resistance			Protease s producti on	conjugatio n Frequency
		Ap	Sm	Rif		
P.aeruginosa P9		+	+	-	-	
	E.coli P9	+	+	+	-	$88x10^{-2}$
P.aeruginosa P15		+	+	-	-	
	E.coli P15	+	+	+	-	$455x10^{-3}$
P.aeruginosa P18		+	+	-	+	
	E.coli P18	No Transconjugant colonies				
P.aeruginosa P22		+	+	-	+	
	E.coli P22	+	+	+	-	$122x10^{-2}$

P.aeruginosa P26		+	+	-	+	
	E.coli P26	+	+	+	-	$154x10^{-2}$

.The *E.coli* MM294 Rif^r recipient cell concentration was 2 x 10^6 cell/ml *
,The symbols (+): Resistance to Antibiotics & proteases producer *
.Sensitive to Antibiotics & non proteases producer :(-)
.Ap: ampicillin, Rif: rifampicin, Sm: streptomycin *

Pigment production was not observed in transconjuagnt colonies when plated on nutrient agar supplemented with Rif and Sm or Am and Sm as a genetic marker. Therefore the transconjugant colonies were screened for pyocyanin pigment production by transferring some colonies of transconjugant colonies approximately 20 colonies, belonging to each conjugation cross on minimal agar medium supplemented with glucose and final concentration of Amp or without and incubated at 37°C for 24 .hours

We observed the pigment production by all tested transconjugants either in the presence of Amp or without Amp. The production of pyocyanin in the transconjugant colonies could be related to the presence of the plasmid R68.45 carrying the genes encoding Amp, Gm and Tc resistances. This plasmid is available in the *P. aeruginosa* isolates which can be transmissible to the genes responsible for pigment production together with Lin resistance gene which may be adjacent to the position of integration of this plasmid in the chromosome, and transferred to the *E.coli* MM294 strain (Hardy, 1986). After screening and plating all transconjugant colonies on skimmed milk agar the results revealed that all transconjugant colonies were unable to produce proteases; these observations may be due to the protease producing genes encoded by chromosomal DNA and this finding agrees with that indicated by Michael *et al*. .((1991



.Fig. (3): The plasmid profile of the transconjugant *E. coli* MM294

The DNA plasmid extracted by alkaline lyses (Kado and Liu, 1981) and migrated on agarose gel 0.7%, 50 volt, for 6hr.

Lane 1: DNA content of *P. aeruginosa* P22 isolate

Lane 2: DNA content of transconjugant *E.coli* P22 that contain conjugative plasmid.

Lane 3: DNA content of standard plasmidless strain *E.coli* MM294.

Lane 4: DNA content of *P. aeruginosa* P9 isolate.

Lane 5: DNA content of transconjugant *E.coli* P9 that contain conjugative plasmids.

Lane 6: DNA content of standard plasmidless strain *E.coli* MM294.

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