Isolation of a Novel Antibiotic Resistance Plasmid DNA from Hospital Isolates of *Pseudomonas aeruginosa*

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**Abstract**

Emergence and dissemination of antibiotic resistance plasmids are major concern for hospital care system and increases the cost and decreases effectiveness of available antibiotics used in treatment of hospitalized patients. In this study two *Pseudomonas aeruginosa*, two *Escherichia coli*, and a *Klebsiella pneumoniae* were isolated from Intensive Care Unit (ICU) of a university hospital, in Kerman, Iran. *K. pneumoniae* exhibited resistance to all antibiotics routinely used in our hospital for treatment of patients except meropenem, while the other isolates were sensitive to carbapenems and ciprofloxacin. Plasmid analysis of the selected isolates showed the presence of a single plasmid with molecular weight 65Kb in the *P. aeruginosa* isolate1. The plasmid was named as pKUM and belonged to incompatibility group -2 (IncP-2). Conjugation by filter mating revealed that resistance associated with gentamicin, kanamycin, cefotaxime, and ceftazidime phenotypes were transferred to *E. coli* ATCC25922 (Rif) recipient cells at the frequencies of 3.13 × 10^-5 and 5.3 ×10^-7 respectively. The results were further supported by curing and transformation experiments. MIC to cefepime was ≥30 µg/mL both in donor as well as the transconjugants while decreased to 0.5µg/mL in cured derivative. The plasmid pKUM was quite stable (86%) in both donor cells and the recipient. From above results we concluded that resistances to third generation of cephalosporins, aminoglycosides and cefepime in *P. aeruginosa* isolate1 were indeed encoded by a conjugal plasmid. Acquisition of cefepime resistance through plasmid complicates the therapy of neutropenic patients in ICU and increases the cost and mortality of these patients.

**Keywords:** *Pseudomonas aeruginosa*: Antibiotic resistance; Plasmid; Conjugation; Transformation; Curing

**Introduction**

*Pseudomonas aeruginosa* is gram negative short rod belong to family Pseudomonaceae. It is motile, oxidase positive, non-spor forming bacteria and grows on simple as well as complex medium. *P. aeruginosa* occupy very important position as nosocomial pathogen and cause serious infections like fulminative septicemia, meningitis or pneumonia in patients hospitalized in the hospitals across the world. This organism is also responsible with high mortality and morbidity in patients with impaired immune system and cystic fibrosis [1].

Antibiotic resistance associated with hospital isolates of *P. aeruginosa* created serious health care concerns particularly in the ICU where seriously ill patients are hospitalized [2]. Multiple Drug Resistance (MDR) in *P. aeruginosa* is defined as the resistance to 3 or 4 of the following antibiotic classes; penicillins, cephalosporins, monobactams, carbapenems, aminoglycosides, and quinolones. These strains constantly cumulate several resistance mechanisms as a consequence of multiple genetic events such as chromosomal mutations or horizontal transfers of resistance genes [3, 4]. Some of these are widely prevalent in southern Europe, Turkey, and Southeast Asia [1].

Different plasmids have been reported in this bacterium mediate resistance to third generation of cephalosporins and penicillins through β- lactamase enzymes [5]. Cefepime has an extended spectrum of activity against Gram-positive and Gram-negative bacteria than third generation agents. Cefepime is usually reserved to treat moderate to severe nosocomial pneumonia, infections caused by multiple antibiotic resistant *P. aeruginosa* and empirical treatment of febrile neutropenia [6].

Similarly, most studies have indicated that around 10% of *P. aeruginosa* isolates are aminoglycosides resistant [7]. Plasmids have been reported to carry genes for enzyme aminoglycosides, acetyltransferase and phosphotransferase [7,8]. In one investigation two clinical isolates of *P. aeruginosa*, was found to transfer gentamicin resistance to other *Pseudomonas* by conjugation rate of 1 × 10^-3, but not to *E. coli* or other enterobacteriaceae [9].

Antibiotic resistance phenotypes and plasmid content of 35 multiple drug resistant *P. aeruginosa* strains were showed that 10 isolates exhibited high level resistance to both gentamicin and tobramycin [10]. Briand et al., [11] isolated a plasmid was transferable to *P. aeruginosa* with a transfer frequency between 10^-5 to 10^-7 per recipient strains and also to *E. coli* K12.

The resistant pattern and antibiotic susceptibility of *P. aeruginosa* have been changing over the past years particularly, in ICU and surgical wounds [12]. A study was undertaken to characterise *P. aeruginosa* strains isolated from burned patients in Tehran, Iran indicated 98% isolated strains were resistant to cefoxitin, 97% to cepotan, 93% to ticarcillin, 89% to ticarcillin/clavulanic acid, 76% to gentamicin and imipenem, 63% to piperacillin, 49% to tetracycline, and 20% to meropenem [13].

In previous study we reported emergence of ciprofloxacin resistance phenotype among *P. aeruginosa* isolated from burn patients [14]. In this investigation we isolated an antibiotic resistant plasmid in *P. aeruginosa* carried resistance to third generation of cephalosporins; cefepime and aminoglycosides. This organism is a major concern...
in nosocomial infections and should therefore be monitored in surveillance studies.

Materials and Methods

Bacterial source and identification

Five multiple drug resistance nosocomial pathogens were isolated from ICU patients hospitalized in Afzalipoor hospital, Kerman, Iran. Patients were either admitted directly to the ICU or transferred from other wards, namely surgery, obstetrics and pediatric wards. Post-operative patients requiring ventilation were admitted to the critical care unit, while patients with medical conditions necessitating ventilation were admitted to the ICU. The patients showed sign such as high fever, bacterimia and sever chill. Samples were collected from blood of each patient and inoculated in to 5 mL Stuart Transport medium (ST) and transferred to microbiology laboratory for further analysis. 200 µL of the samples were inoculated onto MacConkey and 5% sheep blood agar medium (Merck, Germany) and identified according to standard biochemical tests [2]. The identified isolates were mixed with 40% glycerol in True North Cryogenic Vials (TNC) containing 1mL sterile Trypticase Soy Broth (TSB) and preserved at -70°C for further investigation.

Antibiotic susceptibility

Antibiotic sensitivity of above isolates was determined by disk diffusion method of Bauer et al., [15] on Mueller-Hinton agar [MHA] (Hi-Media, India) using commercially available paper disks (Padtan-Tebr, Iran). Antibiotics both anti-pseudomonal and non-anti-pseudomonal were used in the following concentrations (in µg mL⁻¹): Nalidixic acid (NA) [30 µg], Cefazidime (CAZ) [30 µg], Imipenem (IPM) [10 µg], sulfamethoxazole (SXT) [10 µg], Ciprofloxacin (CP) [5 µg], Tetracycline (Te) [30 µg], Chloramphenicol (C) [30 µg], Amoxicillin (AMX) [25 µg], Cefotaxime (CTX) [30 µg], CAZ + clavulanic acid (CZA) [30 µg + 10 µg], Gentamicin (Gm) [10 µg], Amikacin (AN) [30 µg] and Kanamycin (Km) [10 µg]. Zone of inhibition surrounding each disk was measured and labeled as resistance, intermediate, sensitive according to CLSI procedure [16,17]. In case of E-test, inoculums preparation and plating, strip application, and subsequent minimum inhibitory concentration (MIC) determinations were carried out in accordance with the manufacturer’s instructions and CLSI guidelines. E. coli ATCC25922 was included as a control strain for susceptibility testing.

Development of rifampicin resistant mutants

For induction of rifampicin mutation, 1mL of the overnight culture of E. coli ATCC25922 has spread throughout nutrient agar plate containing gradient concentration of rifampicin (Sigma grade) and incubated at 37 °C for 2 days. The colonies grown on the highest gradient concentration of the rifampicin were selected and re-steaked on Muller-Hinton agar plate supplemented with 100 µg/mL rifampicin. Mutant strain was designated as E. coli ATCC25922 (Rif'). The rifampicin was selected because the resistant gene located on the chromosome and all isolates were susceptible to this antibiotic. The geneunity of the mutants was confirmed by existence of metallic sheen on EMB plate.

Plasmid DNA extraction

Extraction of the plasmid DNA from all isolates was carried out using alkaline lysis method [18] and observed on 0.7% agarose gel (Merck-Germany). Electrophoresis was conducted in horizontal bed apparatus for 3 hours at 60 mA using 500 mL 1 mM Tris- Borate- EDTA (TBE × 1) buffer (pH-8.3). Plasmid bands were photographed by a camera attached UV gel documentation system (UV Tech- Cambridge) after stained with 0.5 µg/mL ethidium bromide for 5 minutes. The molecular weight of the plasmid was determined by running λ phage DNA as ladder digested with HindIII restriction enzyme.

Conjugation and plasmid transformation

Conjugation between P. aeruginosa isolate1 as donor and rifampicin-resistant E. coli ATCC 25922 (Rif') as recipient cells was carried out by membrane filter technique as described previously [19]. The transconjugants were selected on Muller – Hinton agar medium containing CTX (30 µg/mL) + Rif (100 µg/mL) and Gm (30 µg/mL) + Rif (100 µg/mL). Conjugation frequency was calculated as the number of transconjugants divided by the recipient cells multiply dilution factor. Simultaneously, a control of conjugation was carried out along side of the test.

Transformation procedure was adopted as described by Sambrook et al., [18] with some modification. Briefly, an E. coli ATCC25922 cell was made recombinant deficient (recA-) by exposing cells to sub-inhibitory concentration of ethidium bromide (0.25 µg/mL) and UV irradiation at 260nm as described by Clark [20]. The cells were made competent for transformation by addition of 200µg of 50 mM ice cold calcium chloride (CaCl₂) [Merck, Germany] to 500 µL of log phase bacterial suspension for 30 minutes on ice powder. The suspension was transferred to a microfuge tube and centrifuged at 8000 rpm for 10 minutes. The cell pellet was resuspended in 200 µL of 50 mM ice cold CaCl₂ followed by addition of 400 µL extracted of plasmid. Microfuge tubes incubated for additional 5min in laboratory temperature. 100 µL of the transformed cells were spread onto selective medium (MHA containing 100 µg/mL CTX and 30 µg/mL Gm separately) and on nonselective medium. The petri plates incubated overnight at 37°C and colonies were checked for presence of the plasmid.

Plasmid curing and stability

Curing experiments were performed using ciprofloxacin, SDS, Eucalysputus plant extract and two temperature 42°C and 44°C as curing agents. Briefly, overnight culture of P. aeruginosa isolate 1 was grown in presence of sub-inhibitory concentration of curing agent for 24 hours at 37°C. A loopful of the organism was streaked on nonselective MHA medium containing antibiotics (CAZ 100 µg/mL & Gm 30 µg/mL) and incubated at 42°C and 44°C separately for 48 hours. The individual colonies were inoculated and incubate at the same temperatures. The colonies from all curing agents were then replica plated by sterile toothpick on MHA medium containing antibiotics (CAZ 100 µg/mL & Gm 30 µg/mL) and on nonselective medium. The colonies that failed to grow on selective medium were considered as putative cured derivatives. The physical loss of plasmid in the cured derivatives was confirmed by agarose gel electrophoresis. The percentage curing was estimated as number of colonies with cured phenotype per 100 colonies tested. Similarly, stability of the plasmid was investigated in presence and absence of antibiotic CTX at intervals of 1, 2, 4, 6 and 24 hours for 1000 generation as described by Lee et al. [21].

Plasmid incompatibility

Plasmid incompatibility was carried out by method of Eaton and Rawlings [22]. Plasmid containing E. coli ATCC25922 (Rif') cells were transformed with a second plasmid belong to IncP, IncW, IncQ and IncC (received from institute pasture, Iran branch) and plated on

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nutrient agar plates with antibiotic selection for both plasmids. Single colonies were inoculated in to 20 mL sterile nutrient broth incubated at 37°C for 24 hours. Survival of the plasmids was then tested by removing selection for both plasmids and growing the cells in 5mL nutrient broth for 100 generations, with transfer of approximately 1,000 cells to fresh medium at 20 generation intervals. Finally, 70 colonies were replica plated to antibiotic containing nutrient agar plates to score for plasmid retention. Cells containing individual plasmids were similarly grown and plated as a control to account for spontaneous plasmid loss.

### Results and Discussion

Over all resistance patterns of the ICU isolates showed that, *P. aeruginosa* isolate1 had broad spectrum resistance to all antibiotics routinely used in our hospital for treatment of patients except imipenem, meropenem, ciprofloxacin, chloramphenicol and it was intermediate to tetracycline (Table 1). It also exhibited MIC >5 µg/mL to piperacillin/tazobactam and >30 to ceftazidime (Table 2). The isolate was also resistant to amikacin and ceftazidime + clavulanic acid. *P. aeruginosa* isolate2 was susceptible tomeropenem, imipenem, chloramphenicol and tetracycline. The reason behind of chloramphenicol sensitivity of both *P. aeruginosa* strains might be due to discontinued administration of this antibiotic in our ICU. In case of *K. pneumoniae*, it was only sensitive to meropenem, tetracycline and resistant to all 14 antibiotics tested. Similarly *E. coli* isolate1 and *E. coli* isolate2 were both sensitive to nalidixic acid, imipenem, meropenem, piperacillin/tazobactam, amikacin and ciprofloxacin. In addition, *E. coli* isolate2 was sensitive to ceftazidime and gentamicin and intermediate to kanamycin (Table 1). Our data indicated that the MDR strains of bacteria are dominant in our ICU and supported the administration of the antibiotics with high resistant phenotype to the recipient. Co-transfer study of antibiotic resistant phenotypes in the transconjugants showed that CAZ, and Km resistant phenotype were co-transferred along with CTX and Gm (Table 3). However, other nosocomial pathogens could not transfer any resistant gene to the above recipient. Co- transfer study of antibiotic resistant phenotypes to the transformants showed that resistance to amikacin and ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin, samples, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to pefloxacin, 58% to pefloxacin eflornithine and 35% to gentamicin [23].

Conjugation between our ICU isolates as donors and *E. coli* ATCC 25922 Rif as a recipient revealed that only *P. aeruginosa* isolate1 could transferred CTX and Gm resistant phenotype to the recipient samples, with frequency of 3.13 × 10^{-5} and 3.2 × 10^{-5} respectively (Table 3), however, other nosocomial pathogens could not transfer any resistant gene to the above recipient. Co- transfer study of antibiotic resistant phenotypes to the transconjugants showed that 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin, samples, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin, samples, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin, samples, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin, samples, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin, samples, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin, samples, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin, samples, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin, samples, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin, samples, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin, samples, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin, samples, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin, samples, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin, samples, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin, samples, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin, samples, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin, samples, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin, samples, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin, samples, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant to tetracycline, while remained resistant to carbenicillin, clindamycin.
ampicillin and co-trimoxazole. Isolation of P. aeruginosa strain from pulmonary brush of a patient hospitalized in a suburb of Paris, France [28] revealed that plasmid was transferred by conjugation to rifampin resistant P. aeruginosa pU21 at a frequency of $2 \times 10^{-7}$ but not to rifampin resistant E. coli K-12 C600.

Plasmid incompatibility and stability revealed that pKUM plasmid was belong to IncP-2 group and was quite stable in both donor cells as well as transconjugants. The stability of the plasmid in P. aeruginosa isolate1 was significantly high in absence of CTX in the beginning of the experiment (99%) however; it decreased to 72% after 24 hours of incubation. In presence of CTX, the stability of pKUM plasmid decreased steadily from 86% to 69% (Figure 2).

Previous studies of antibiotic susceptibility among hospital isolates

| Donor cells | Recipient cells | Selective medium | Conjugation frequency | phenotype Co-transferred |
|-------------|-----------------|------------------|----------------------|-------------------------|
| *P. aeruginosa* isolate1 | *E. coli* ATCC 25922 (Rif *) | MHA* + CTX(30µg/mL) & Rif(100µg/mL) & Gm(30µg/mL) Rif(100µg/mL) | $3.13 \times 10^{-5}$ | CAZ, Km |

*MHA: Muller – Hinton agar.

A control for conjugation was carried out and it was $1 \times 10^{-9}$.

Conjugation was carried out by membrane filter technique with ratio of 2:1 for donor and recipient cells.

Table 3: Conjugation between multiple drug resistance P. aeruginosa isolate 1 as donor and E. coli ATCC 25922(Rif) as recipient by membrane filter method.

![Figure 1: Agarose gel electrophoresis of the plasmid pKUM isolated from P. aeruginosa isolate1.](image)

![Figure 2: Stability of plasmid pKUM in P. aeruginosa isolate1 in presence and absence of selection in different time intervals.](image)

Figure 2: Stability of plasmid pKUM in P. aeruginosa isolate1 in presence and absence of selection in different time intervals. TSB=Trypticase Soy Broth, CTX= Cefotaxime.

in Kerman indicated sensitivity of isolated bacteria to third generation of cephalosporins, carbapenems and ciprofloxacin [29], however, emergence and dissemination of resistance to imipenem, cefepime and ciprofloxacin in our hospital through horizontal gene transfer must be prevented by routine antibiotic surveillance so as to maximize the possibility of administering an effective therapeutic regime whenever there is a need.

Conclusion

As the above results indicated, it can be concluded that among ICU isolates, only *P. aeruginosa* isolate1 was carried a MDR plasmid. The unique features of the pKUM plasmid were different resistances genes encoded, high stability, low curing efficiency and easy transferred through conjugation to the other nosocomial pathogens. The acquisition of cefepime, third generation of cephalosporins and aminoglycosides resistances through plasmid may complicate the therapy of the patients in ICU and increase the cost, mortality and prolonging hospitalization in the hospital.

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