

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^r) 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 $\geq 30 \mu\text{g/mL}$ both in donor as well as the transconjugants while decreased to $0.5 \mu\text{g/mL}$ 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 conjugative 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 Pseudomonadaceae. It is motile, oxidase positive, non-spore 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 fulminant 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 cefotetan, 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

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Received May 30, 2013; Accepted June 14, 2013; Published June 16, 2013

Citation: Jafari E, Shakibaie MR, Poormasoomi L (2013) Isolation of a Novel Antibiotic Resistance Plasmid DNA from Hospital Isolates of *Pseudomonas aeruginosa*. J Clin Exp Pathol 3: 140. doi:10.4172/2161-0681.1000140

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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 signs such as high fever, bacteremia and severe chills. Samples were collected from blood of each patient and inoculated into 5 mL Stuart Transport medium (ST) and transferred to microbiology laboratory for further analysis. 200 μ L 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 1 mL 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-Teb, Iran). Antibiotics both anti-pseudomonal and non-anti-pseudomonal were used in the following concentrations (in μ g mL⁻¹): Nalidixic acid (NA) [30 μ g], Ceftazidime (CAZ) [30 μ g], Imipenem (IPM) [10 μ g], sulfamethoxazole (SXT) [10 μ g], Ciprofloxacin (CP) [5 μ g], Tetracycline (Te) [30 μ g], Chloramphenicol (C) [30 μ g], Amoxicillin (AMX) [25 μ g], Cefotaxime (CTX) [30 μ g], CAZ + clavulanic acid (CZA) [30 μ g + 10 μ g], Gentamicin (Gm) [10 μ g], Amikacin (AN) [30 μ g] and Kanamycin (Km) [10 μ g]. 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, inoculum 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, 1 mL of the overnight culture of *E. coli* ATCC25922 was 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-streaked on Muller-Hinton agar plate supplemented with 100 μ g/mL rifampicin. Mutant strain was designated as *E. coli* ATCC25922 (Rif^r). The rifampicin was selected because the resistant gene located on the chromosome and all isolates were susceptible to this antibiotic. The genuinity 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 1mM Tris- Borate- EDTA

(TBE \times 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 μ g/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* isolate 1 as donor and rifampicin-resistant *E. coli* ATCC 25922 (Rif^r) 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 μ g/mL) + Rif (100 μ g/mL) and Gm (30 μ g/mL) + Rif (100 μ g/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 μ g/mL) and UV irradiation at 260nm as described by Clark [20]. The cells were made competent for transformation by addition of 200 μ L of 50 mM ice cold calcium chloride (CaCl₂) [Merck, Germany] to 500 μ L 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 μ L of 50 mM ice cold CaCl₂ followed by addition of 400 μ L extracted of plasmid. Microfuge tubes incubated for additional 5min in laboratory temperature. 100 μ L of the transformed cells were spread onto selective medium (MHA containing 100 μ g/mL CTX and 30 μ g/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, Eucalyptus 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 agents for 24 hours at 37°C. A loopful of the organism from the highest concentration of curing agents streaked on MacConkey agar to obtain isolated colonies. In case of high temperature, the overnight growth of the organism was streaked on nutrient agar plates and incubated at 42°C and 44°C separately for 48 hours. The individual colonies were inoculated and incubated at the same temperatures. The colonies from all curing agents were then replica plated by sterile toothpick on MHA medium containing antibiotics (CAZ 100 μ g/mL & Gm 30 μ g/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^r) cells were transformed with a second plasmid belong to IncP, IncW, IncQ and IncC (received from institute pasture, Iran branch) and plated on

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 cefepime (Table 2). The isolate was also resistant to amikacin and ceftazidime + clavulanic acid. *P. aeruginosa* isolate2 was susceptible to meropenem, 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 antipseudomonal activity, particularly ciprofloxacin and carbapenems. The other antibiotics especially third generation of cephalosporins, cefepime and aminoglycosides were ineffective in routine therapy of infected patients in our hospital.

Many researches were conducted across the globe on emergence of multiple drug resistance *P. aeruginosa* strains isolated from clinical

Antibiotic	<i>P.aeruginosa</i> isolate 1	<i>P.aeruginosa</i> isolate 2	<i>K. pneumoniae</i>	<i>E.coli</i> isolate 1	<i>E.coli</i> isolate 2	<i>E.coli</i> ATCC 25922
NA	R	R	R	S	S	S
CAZ	R	R	R	R	S	S
MEN	S	S	S	S	S	S
IPM	S	S	R	S	S	S
SXT	R	R	R	R	I	S
CP	S	R	R	S	S	S
Te	I	S	S	R	R	S
AMX	R	R	R	R	R	R
CTX	R	R	R	R	R	S
Gm	R	R	R	R	S	S
Km	R	R	R	R	I	S
C	S	S	R	I	S	S
CPM	R	ND	ND	ND	ND	S
PIT	R	R	R	S	S	S

Mueller – Hinton agar was used for antimicrobial disk diffusion susceptibility testing. CFU was adjusted to 10⁶
 NA: Nalidixic Acid; CAZ: Ceftazidime; MEN: Meropenem; IPM: Imipenem; SXT: Trimethoprim; CP: Ciprofloxacin; Te: Tetracycline; AMX: Amoxicillin; CTX: Cefotaxime; Gm: Gentamicin; Km: Kanamycin; C: Chloramphenicol; PIT: Piperacillin/Tazobactam; CPM: Cefepime
 S: Sensitive; I: Intermediate; R: Resistant; ND: Not determined

Table 1: Antibiotic resistance pattern of nosocomial bacteria to 14 antibiotics isolated from ICU patients hospitalized in the Afzalipoor hospital, Kerman, Iran.

Antibiotic	<i>P.aeruginosa</i> isolate1	MIC(µg/mL) <i>E.coli</i> transconjugants	<i>P.aeruginosa</i> Plasmid Cured
C	0.5	0.5	0.5
Gm	>5	>5	0.1
PIT	>5	>5	0.5
CPM	>30	>30	1.0

C: Chloramphenicol; Gm: Gentamicin; PIT: Piperacillin / Tazobactam; CPM: Cefepime;
 MIC: Minimum Inhibitory Concentration;
 The inoculum size was 10⁸ CFU/mL.
 Muller-Hinton agar was used for MIC determination.

Table 2: Minimum Inhibitory Concentration (MIC) of four antibiotics against *P.aeruginosa* isolate1, *E.coli*ATCC25922 (Rif^r) transconjugants and plasmid cured derivative.

samples, in one study 94% were susceptible to imipenem, 90.2% to ciprofloxacin, 89% to amikacin and 78% to ceftazidime but 82% were resistant ofloxacin, 58% to pefloxacin and 35% to gentamicin [23].

Conjugation between our ICU isolates as donors and *E. coli* ATCC 25922 Rif^r as a recipient revealed that only *P. aeruginosa* isolate1 could transferred CTX and Gm resistant phenotype to the recipient cells 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 in the transconjugants showed that CAZ, and Km resistant phenotypes were co-transferred along with CTX and Gm (Table 3). Furthermore, MIC to CPM and PTI increased from 0.5 to 30 and >5 µg/mL respectively *E. coli* ATCC 25922 Rif^r transconjugants exhibited following antibiotic susceptibility pattern by disk diffusion; SXT^s, CP^s, IPM^s, NA^s, Te^s, Gm^r, CAZ^r, CPM^r, CTX^r and Rif^r. Our data are in agreement with work carried out previously by the other authors [24,25].

To confirm presence of the plasmid in both *P. aeruginosa* isolate1 and the *E. coli* ATCC 25922 Rif^r transconjugants, we performed plasmid isolation and subsequent agarose gel electrophoresis. It was found that both organisms carried 65 Kb plasmid band (Figure 1). The isolated plasmid was designated as pKUM.

Plasmid pKUM was further transformed to recA⁻ *E. coli* ATCC 25922 with a frequency of 5.3×10⁻⁷ as shown in (Table 4) Transformation resulted in simultaneous transfer of CTX, Gm, Km and CAZ resistant phenotypes to the transformants. This data were further supported by existence of a plasmid band in the transformants (Figure 1). Transfer of these resistant genes creates serious challenge for physician in treating ICU patients. Similarly, Our data showed that conjugative plasmid in nosocomial bacteria especially in ICU can easily transferred to unrelated species and may resulted in emergence of pan-resistant strains, where no longer any antibiotics could be used. This may create huge problem for low income countries in Africa or Latin America [26].

Curing of plasmid pKUM showed that, SDS (400 µg/mL) had highest percentage of the curing activity (1.38%), while exposing cells to temperature 42°C did not cure the plasmid at all. Eucalyptus extract and ciprofloxacin could cure plasmid with 1.25 and 1.19% respectively. Similarly, loss of antibiotic resistance at 44°C was 1.13%. The curing of the antibiotic resistance phenotypes was indeed associated with physical loss of plasmid on agarose gel as shown in Figure 1.

In one study in India, ethidium bromide was used for plasmid curing [27]. Curing and transformation experiments showed that resistance to amikacin was plasmid mediated. It was noted that the isolates that lost plasmids became susceptible to amikacin, chloramphenicol and tetracycline, while remained resistant to carbencillin, 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 2×10^{-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

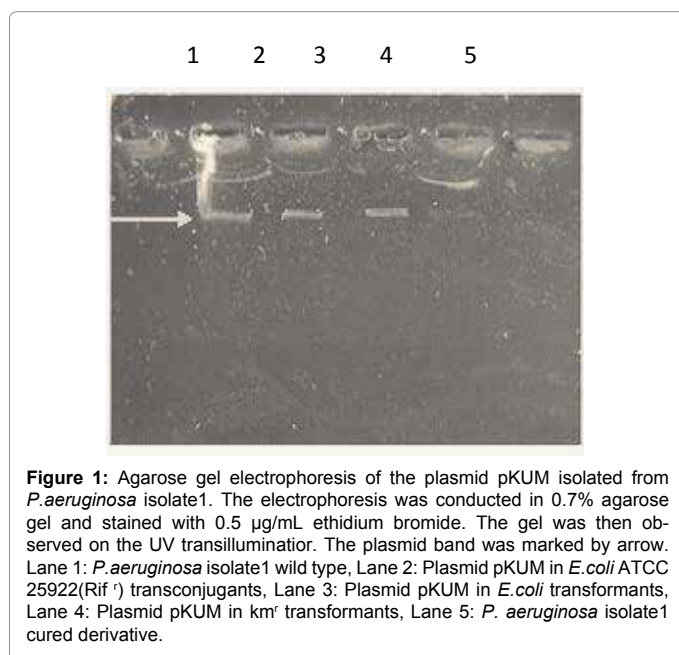
Donor cells	Recipient cells	Selective medium	Conjugation frequency	phenotype Co-transferred
<i>P. aeruginosa</i> isolate1 PIT ^r , CAZ ^r , CPM ^r , CTX ^r , Gm ^r , Km ^r	<i>E. coli</i> ATCC 25922 (Rif ^r)	MHA* + CTX(30µg/mL) Rif(100µg/mL) & Gm(30µg/mL) Rif(100µg/mL)	3.13×10^{-5} 3.2×10^{-5}	CAZ, Km

*MHA: Muller – Hinton agar.

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

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

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



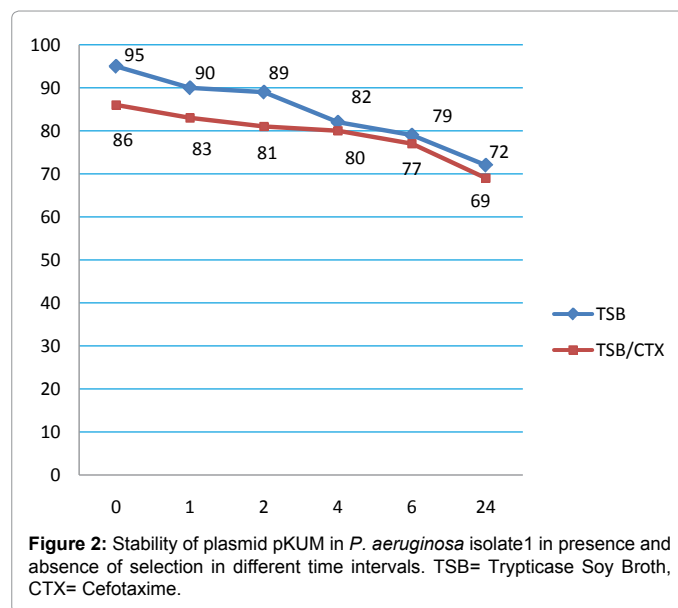
Recipient	Resistant phenotype	Selective medium	Resistance TF Co-transformed
<i>E. coli</i> ATCC25922 recA ⁻	CTX ^r , Gm ^r	MHA + CTX(30µg/mL) + Gm(10µg/mL)	5.3×10^{-7} Km, CAZ

Competent cells were generated by addition of 50mM ice cold CaCl₂.

TF: Transformation Frequency per recipient cells.

Recombinant negative strain was obtained by addition of ethidium bromide to the log phase bacterial cells.

Table 4: Transformation of antibiotic resistance plasmid pKUM to *E. coli* ATCC 25922.



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 pathogen. 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.

Acknowledgements

Authors would like to thank the staff of Department of Microbiology, Kerman University of Medical Sciences for help and providing facilities for this research.

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Citation: Jafar E, Shakibaie MR, Poormasoomi L (2013) Isolation of a Novel Antibiotic Resistance Plasmid DNA from Hospital Isolates of *Pseudomonas aeruginosa*. J Clin Exp Pathol 3: 140. doi:10.4172/2161-0681.1000140

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