International Scholars Journals

Advances in Food Science and Technology ISSN: 6732-4215 Vol. 5 (12), pp. 001-007, December, 2017. Available online at www.internationalscholarsjournals.org © International Scholars Journals



*Full Length Research Paper*

# The role of plasmid in multidrug resistance P. aeruginosa clinical isolates from burn infections

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Accepted 20 January, 2016

**Twenty-one isolates of *Pseudomonas aeruginosa* were collected from burns hospital laboratories in Duhok City. They were identified using the API 20E system, biochemical and morphological characteristics. All isolates had multiple resistance to ampicillin, tetracycline, rifampicin, amoxicillin, chloramphenicol, cefotaxime, erythromycin, penicillin, neomycin, streptomycin, trimethoprim and gentamycin, and were sensitive to ciprofloxacin. The minimum inhibitory concentrations (MICs) of two curing agents, sodium dodecyl sulphate (SDS) and ethidium bromide, used in this study were determined and the results indicated that the curing percentage and efficiency of each curing agent was determined. From treatment with 700 µg/ml ethidium bromide, it was observed that no cured cells were obtained for all antibiotics used, whereas in the treatment done for three incubation times using SDS at a concentration of 1% (W/V) with different incubation periods as curing agent, the bacterial colonies that lost their antibiotics resistance appeared with different curing rates. The results showed 75 - 100% of curing for streptomycin and gentamycin with little effect on erythromycin and trimethoprim (0 - 8%), while SDS had variable effect on other antibiotics resistance in some isolates. In addition, curing of plasmid DNA by elevated temperature (45°C) was carried out and the results revealed high percentage of curing for all antibiotics resistance as they were compared with that induced by the action of SDS and ethidium bromide. The percentages of bacterial colonies that lost their antibiotic resistance were 55 - 100% for curing to all antibiotic resistance in the six tested isolates.**

**Key words:** *Pseudomonas aeruginosa,* multidrug resistance, plasmid curing.

## INTRODUCTION

*Pseudomonas aeruginosa* is currently one of the most frequent nosocomial pathogens and the infections caused by this organism are often difficult to treat due to antibiotic resistance (Emori and Gaynes, 1993) The mechanisms of resistance to antibiotics include reduced cell wall permeability, production of chromosomal and plasmid mediated β-lactamases (Livermore, 1989), aminoglycoside-modifying enzymes (Prince, 1986) and an active multidrug efflux mechanism (Li et al., 1994).

*P. aeruginosa* has been increasingly recognized for its ability to cause significant hospital-associated outbreaks of infection, particularly with the emergence of multidrug resistant strains. Outbreaks of multidrug-resistant *P. aeruginosa* colonization or infection have been reported on urology wards, a burn unit, hematology/oncology units, and adult and neonatal critical care units (Gillespie et al., 2000; Douglas et al., 2001).

Drug resistance is an alarming problem worldwide and is spreading rapidly due to overuse, self medication, and non-therapeutic use of antimicrobials (Slama et al., 2005). Antimicrobials themselves act as a selective pressure which allows the growth of resistant bacteria within a population and inhibits susceptible bacteria (Levy, 1994). Drug resistance property in bacteria is usually born in R-plasmids, which can be disseminated to diverse population and regions causing worldwide problems. R-plasmids from resistant strains of an organism may transfer to a sensitive counterpart, which can show the same drug resistance in the donor strain (Brüssow et al., 2004). An increase in the emergence of multi-drug resistant bacteria in recent years is worrying, though the presence of antibiotic resistant genes on bacterial plasmids has further helped in the transmission

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and spread of drug resistance among pathogenic bacteria (Zulkifli et al., 2009).

Emergence of multidrug resistance in pathogenic bacteria has created immense clinical problem in the treatment of infectious disease. Elimination of plasmid DNA mediated antibiotic resistance in pathogenic bacteria is of great practical significance both in chemotherapy of bacteria and in microbial genetics. Elimination of plasmid DNA can be performed using different chemical materials including plant extracts (Reuter and Sendel, 1994).

One strategy to minimize plasmid transmission of antibiotic resistance is to eliminate the plasmids. This process is known as 'curing' and many compounds have been shown to be capable of causing this effect (Caro et al., 1984; Trevors, 1386). Curing may occur naturally through cell division or by treating the cells with chemical and physical agents (Snyder and Champness, 1997).

The inhibition of conjugational transfer of antibiotic resistance plasmid can be exploited to reduce the spread of antibiotic resistance plasmid in the ecosystem. Inhibition of plasmid replication at various stages, as shown in the "rolling circle" model (replication, partition, and conjugal transfer) may also be the theoretical basis for the elimination of bacterial virulence in the case of plasmid mediated pathogenicity and antibiotic resistance (Brüssow, 2004). The aim of this study is to shed light on the role of plasmid in multidrug resistance *P. aeruginosa* clinical isolates from burn infections.

## MATERIALS AND METHODS

### Sample collection and identification of bacterial isolates

Twenty-one isolates of *P. aeruginosa* were collected from burns hospital in Duhok City. They were transferred to the laboratory and activated using brain heart infusion broth. After activation and inoculation of the isolates on the MacConKey and blood agar, single colonies were selected for more purification, and oxidase test was done for positive isolates. Microscopically, Gram negative rod shape was identified provisionally as *Pseudomonas aeruginosa*, and subcultured on nutrient agar slants. After incubation at 37°C for 24 h, it was stored at 4°C, till other bacteriological tests were carried out (Holt et al., 1994). The identification of the selected isolates was confirmed by Analytical Profile Index (API 20E) for Enterobacteriaceae from Biomerex France.

### Preparation of media and stock solutions of antibiotic

Stock solution of each antibiotic was prepared by dissolving each antibiotic in suitable solvent according to the method of Puhler and Timmis (1984) and stored at -20°C until it was used, while the method of Grant and Pittard (1974) was used for preparation of the antibiotic media by adding antibiotic separately to the sterile. The nutrient agar media was allowed to cool down at 45-50°C with final concentration of each antibiotic as described in Table 1.

### Antibiotic susceptibility test

Antibiotics susceptibility patterns of twenty-one isolates were determined with thirteen commercially and widely used antibiotics to confirm the multi-drug resistance of these isolates according to the method of Ahmad (1989). The isolates were placed on streak nutrient agar plates containing the used antibiotics with their final concentration of bacterial isolates, after which the plates were incubated at 37°C overnight and the results of the bacterial growth were recorded.

### Plasmid curing

Curing experiments were performed using sodium dodecyl sulfate (SDS), ethidium bromide and elevated temperature (45°C) as curing agents.

#### *Curing with ethidium bromide*

This method was described by Darfeuille-Michanol et al. (1986), in which the elimination of antibiotics resistance plasmid DNA from *P. aeruginosa* isolates was done by ethidium bromide, as follow: 10 ml of nutrient broth containing 700 µg/ml ethidium bromide was inoculated with 0.3 ml of overnight culture of *P. aeruginosa* isolates, and incubated at 37°C for 24, 48 and 72 h. Serial dilution was performed up to $10^{-7}$ by 0.1 ml of interval incubated samples, and 0.1 ml of the last three dilutions was placed on nutrient agar plates, then all plates were incubated at 37°C for 24 h. Several colonies were transferred from the plates for plasmid DNA extraction and electrophoresis was achieved to observe the loss of the plasmids by gel electrophoresis techniques.

#### *Curing of plasmid by sodium dodecyl sulfate*

Plasmid curing by SDS was done by the method described by Tomoeda et al. (1974) as follows: a test tube containing 5 ml of nutrient broth was prepared with addition of the appropriate antibiotic at a final concentration (50 µg/ml), then inoculated with single colony of *P. aeruginosa* isolates, and incubated at 37°C for 24 h. The dilution was prepared up to $10^{-3}$ dilution by nutrient broth containing 0.05, 0.1, 0.25, 0.5, 1, 2 and 2.5% (W/V) (SDS), then the third dilution was incubated at 37°C for 24 h. Serial dilutions were prepared up to $10^{-6}$, then 0.1 ml of the last three dilutions were spread on nutrient agar plates that contained appropriate antibiotics and incubated at 37°C for 24 h, until the result appeared.

#### *Plasmid curing by physical agents (elevated temperature)*

A single colony of *P. aeruginosa* isolate was inoculated

**Table 1.** The groups of *p. aeruginosa* isolates and their Sensitivity and resistance to the antimicrobial used.

| No. of isolates | Nutrient agar plates containing the final concentrations of antibiotics in µg/ml of | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Amp (25) | Tc (30) | Tri (10) | Rif (10) | Ctx (20) | Gm (60) | Cip (25) | Ax (100) | Sm (20) | pi (30) | Neo (10) | Er 30 | Cm (20) |
| P1 | R | R | R | R | R | R | S | R | R | R | S | S | R |
| P2 | R | S | R | S | R | S | S | R | R | S | R | S | S |
| P3 | R | R | R | S | R | S | S | R | R | R | R | S | R |
| P4 | R | R | R | R | R | S | S | R | R | S | S | R | R |
| P5 | R | S | S | R | R | S | R | R | R | S | R | S | R |
| P6 | R | R | R | S | R | R | R | R | R | R | R | R | R |
| P7 | R | R | S | S | S | R | S | R | S | S | S | R | S |
| P8 | R | R | R | R | R | S | S | R | R | R | R | R | R |
| P9 | R | S | R | S | S | S | S | R | R | R | S | S | S |
| P10 | R | R | R | R | R | R | R | R | R | S | R | R | R |
| P11 | R | S | S | R | R | S | R | R | S | R | S | S | R |
| P12 | R | R | S | S | R | R | R | R | S | R | S | R | R |
| P13 | R | S | R | R | R | S | S | R | R | S | S | S | R |
| P14 | R | R | R | S | R | R | R | R | R | R | S | R | R |
| P15 | R | R | S | R | S | R | S | R | S | S | S | S | S |
| P16 | R | S | S | R | R | R | R | R | R | S | S | S | R |
| P17 | R | R | R | S | S | R | S | R | R | R | S | R | R |
| P18 | R | S | S | S | S | S | S | R | R | S | S | S | R |
| P19 | R | R | R | R | R | R | S | R | S | R | S | R | R |
| P20 | R | R | S | R | R | S | S | R | R | S | R | R | R |
| P21 | R | R | R | R | R | R | R | R | R | R | S | R | R |

R: refers to resistance, S: refers to sensitive.
Ap: ampicillin, Tc: tetracycline, Rif: rifampicin, Ax: amoxicillin, Cip: ciprofloxacin, Cm: chloramphenicol, Ctx: cefotaxime, Ery: erythromycin, Pi:penicillin, N:neomycin, Sm: streptomycin, tri: trimethoprim, Gm: gentamycin.

into 10 ml of nutrient broth. After incubation at 37°C for 24 h, 0.2 ml of bacterial culture was inoculated into 10 ml of fresh nutrient broth and incubated at an elevated temperature of 45°C for 24 h with shaking at 100 rpm. More so, several dilutions were performed up to $10^{-7}$ dilutions, then 0.1 ml of the last three dilutions were spread on plates of nutrient agar which contain different antibiotics and incubated at 37°C for 24 h. Subsequently, the results were recorded by the loss of ability of the tested bacteria to survive on the medium which contains the antibacterial agents (Kheder, 2002).

### *Selection of the cured bacterial cells*

In all treatment of curing agents, master plates were prepared containing 100 bacterial treated colonies. Replica plating technique was used in order to determine the cured cells on the nutrient agar plates containing the antibiotics separately for each of the isolate and the untreated cells were used as the control. The results were recorded, and then the cured cells were selected and stored for the next tests, to be sure that the plasmid was cured through comparison with the original strains. However, the plasmid DNA was extracted from cured cells for Agarose gel electrophoresis study (Sambrook et al., 1989).

## RESULTS AND DISCUSSION

### Isolation and identification of *P. aeruginosa*

About twenty-one isolates of *P. aeruginosa* were collected from the laboratory of burns hospital in Duhok City. Cultural and morphological characteristics were used to characterize bacterial isolates by using nutrient, MacConkey's and blood agar. The colonies of *P. aeruginosa* isolates had smooth, large and oval appearance, with flat edge and an elevated appearance. Eighteen of these isolates produced pyocyanin (blue green pigment), which is in accordance with that mentioned by Todar (2004). The bacterial cells from smear preparation are Gram-negative rods, arranged singly or in short chain. All bacterial produced colorless colonies and did not ferment lactose on MacConkey's agar. Furthermore, biochemical tests were performed to support the results. All the isolates were oxidase and catalase positive, which is an important characteristic for all the bacteria. This result is in agreement with the

results found by Ahmad (1989) and Bennett (2008).

**Antibiotic susceptibility patterns of the bacterial isolates**

The results of the antibiotic susceptibility of *P. aeruginosa* isolates included in this study for 13 antimicrobial agents are shown in Table 1. The results of the antibiotic susceptibility test showed higher number of multidrug resistance among *P. aeruginosa* isolates as shown in Table 1. All the isolates showed resistance to ampicillin and amoxicillin, though most isolates were sensitive to ciprofloxacin and neomycin, while others revealed variation in their resistance. The bacterial isolates were grouped according to their resistance to the antibiotics under study into six groups and labeled P1, P6, P8, P10, P14 and P21.

Originally, *P. aeruginosa* harbored R-plasmid encoding multiple antibiotics resistances (Foster, 1983). The organism is generally resistant to numerous antimicrobial agents due to natural resistance in particular impermeability or mutations and acquisition of resistant determinants. Plasmid and integron play a crucial role in acquisition of mobile elements (Japoni et al., 2009).

The results of antibiotic resistance study indicate that majority of *P. aeruginosa* showed antibiotic resistance to one or more antibiotics. Similar results were reported by Japoni et al. (2006) who said almost all *P. aeruginosa* isolated from burn patients were resistant to all tested anti-Pseudomonal antibiotics except carbapenems (meropenem and imipenem). Moreover, several reports from Iran confirmed multidrug resistance of burn isolates (Ekrami and Kalantar, 2007; Estahbanati et al., 2002).

**Plasmid curing**

***Curing by sodium dodecyle sulphate (SDS)***

Curing experiments with different concentrations of SDS (0.1, 0.5, 1 and 2%) (W/V) were performed on the Pseudomonal isolates to determine changes in plasmid content associated with antibiotic resistance pattern. The results obtained, indicated that SDS at concentration of 0.1 and 0.5% had no effect on plasmid curing and complete lysis of treated bacterial cells at 2%, but curing of antibiotics resistance was observed at 1% with different incubation periods.

Table 2 shows the effect of 1% (w/v) SDS as curing agent on the plasmid DNA of *P. aeruginosa* (P1, P6, P8, P10, P14, and P21 isolates) with three incubation times (24, 48, and 72 h). The results illustrated that all bacterial colonies appeared sensitive to streptomycin and gentamycin (75-100%) and maintain their resistance to erythromycin (0-1%) and trimethoprim (3-8%). After 1% of SDS treatment for all treated isolates and during all the incubation times, this study showed that the encoding genes of resistances may be located on the chromosomal DNA. Other antibiotic resistances were affected by the SDS of all isolates with different rates. This result is in agreement with that of Bennett (2008) who found that all isolates of *P. aeruginosa* maintain their resistances to lincomycin and erythromycin after 1% SDS treatment for three incubation periods. This study's result is also in agreement with that of Ahmad (1989) who found that all isolates of *P. aeruginosa* isolated from different human infections appeared 100% sensitive to streptomycin for all isolates after 1% SDS treatment and during all incubation times.

The results of this study revealed that *P. aeruginosa* isolates responded in different rates to 1% SDS, and this may be related to the permeability through outer membrane, and to the location of antibiotic resistant genes which carried different plasmids. Sonstein and Baldwin (1972) elucidate that the effectiveness of SDS may be related to plasmid copy number, or the amount of enzyme which inactivate antibiotics.

Adachi et al. (1972) found that SDS was only effective in elimination of sex (F) and R-plasmids in *E. coli* in a high frequency at a concentration that is higher than 1%. Moreover, it was reported that the longer the incubation times (24 to 72 h), the higher the frequency of sensitivity. In addition, comparing the genus with other genus of bacteria, Saffawi (2001) found that the SDS affected the antibiotic resistant genes in *S. aureus* isolated from different environments. However, these resistances which were cured were conferred by Am, Cm and Sm genes with 100 and 65% on penicillin gene when the SDS was used at a concentration of 0.002% (W/V).

***Curing by ethidium bromide***

Ethidium bromide was used as a curing agent according to the method described by Shahid and Malik (2004). The minimal inhibitory concentration of ethidium bromide was determined for the bacterial isolates in nutrient broth and the highest concentration permitting growth was used for plasmid curing. The results show that 700 µg/ml of ethidium bromide had no effect on curing of the plasmid DNA carrying all the antibiotics resistance genes, for all tested isolates and for different incubation periods (24, 48 and 72 h).

In general, EB affects the plasmid DNA encoding amikacin, carbencillin, and tetracycline resistances with various rates, though the antibiotic resistance genes may be located on low copy number plasmid. This result agrees with that of Keyser et al. (1982) who reported that low copy number plasmid was efficiently cured by EB. The agents causing complete inhibition of plasmid replication like Acridine orange and ethidium bromide intercalate between base pairs in DNA. Furthermore, they suggested that differences in DNA polymerase and RNA polymerase sensitive are responsible for differences in EB sensitivity to bacterial strains due to differences in the

**Table 1.** Show the effect of SDS at concentration 1% on curing the antibiotics resistance from *P. aeruginosa* isolates with three different incubation periods.

| Antibiotics | % of colonies losing their resistance to antibiotics | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 24 h | | | | | | 48 h | | | | | | 72 h | | | | | |
| | P1 | P6 | P8 | P10 | P14 | P21 | P1 | P6 | P8 | P10 | P14 | P21 | P1 | P6 | P8 | P10 | P14 | P21 |
| Amp | 12 | 0 | 8 | 0 | 3 | 12 | 27 | 0 | 0 | 0 | 16 | 20 | 43 | 0 | 0 | 0 | 15 | 27 |
| Tc | 54 | 67 | 13 | 16 | 11 | 27 | 21 | 8 | 9 | 18 | 24 | 40 | 0 | 0 | 32 | 56 | 80 | 15 |
| Rif | 0 | S | 14 | 4 | S | 22 | 0 | S | 0 | 12 | S | 37 | 17 | S | 0 | 0 | S | 25 |
| Cip | S | 53 | S | 26 | 0 | 15 | S | 0 | S | 76 | 44 | 32 | S | 0 | S | 14 | 0 | 13 |
| Ax | 42 | 16 | 29 | 31 | 6 | 21 | 0 | 44 | 11 | 11 | 8 | 10 | 16 | 26 | 40 | 0 | 21 | 35 |
| Cm | 53 | 11 | 13 | 7 | 17 | 47 | 21 | 25 | 17 | 27 | 30 | 0 | 0 | 7 | 0 | 0 | 0 | 0 |
| Ctx | 34 | 27 | 12 | 8 | 17 | 33 | 3 | 43 | 12 | 22 | 32 | 44 | 11 | 2 | 17 | 55 | 32 | 66 |
| Er | S | 0 | 0 | 3 | 1 | 0 | S | 2 | 0 | 0 | 0 | 3 | S | 0 | 0 | 0 | 0 | 0 |
| Pi | 11 | 14 | 9 | S | 10 | 21 | 26 | 11 | 24 | S | 21 | 33 | 37 | 0 | 23 | S | 34 | 54 |
| Neo | S | 91 | 17 | 19 | S | S | S | 34 | 17 | 23 | S | S | S | 0 | 0 | 0 | S | S |
| Sm | 89 | 90 | 83 | 93 | 78 | 78 | 73 | 76 | 77 | 82 | 100 | 100 | 80 | 77 | 84 | 75 | 88 | 79 |
| Tri | 0 | 0 | 0 | 3 | 0 | 8 | 1 | 0 | 3 | 0 | 0 | 5 | 0 | 0 | 0 | 6 | 0 | 4 |
| Gm | 93 | 78 | S | 90 | 81 | 100 | 0 | 83 | S | 75 | 78 | 0 | 100 | 0 | S | 83 | 86 | 100 |

**Table 3.** The effect of elevated temperature (45°C) on curing the antibiotics resistance from *P. aeruginosa* isolates.

| Isolate number | % of colonies losing their resistance to antibiotics | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Amp | Tc | Tri | Rif | Ctx | Gm | Cip | Ax | Sm | pi | Neo | Er | Cm |
| P1 | 78 | 98 | 85 | 66 | 79 | 100 | S | 99 | 88 | 76 | S | S | 75 |
| P6 | 100 | 74 | 100 | S | 100 | 75 | 61 | 100 | 63 | 60 | 100 | 74 | 92 |
| P8 | 98 | 72 | 59 | 97 | 87 | S | S | 82 | 89 | 94 | 95 | 91 | 83 |
| P10 | 100 | 62 | 92 | 97 | 100 | 89 | 100 | 100 | 83 | S | 73 | 88 | 100 |
| P14 | 63 | 55 | 100 | S | 85 | 55 | 99 | 79 | 96 | 79 | S | 100 | 99 |
| P21 | 100 | 83 | 81 | 72 | 92 | 78 | 92 | 82 | 88 | 67 | S | 76 | 75 |

rate of the agent's penetration into different strains of Enterobacteriaceae.

Rubins and Rosenblum (1971) speculated that further exposure to EB caused the rate of elimination to decrease and the resistance to EB to increase, and the resistance levels tended to increase slightly after 24 h of growth in EB. This finding agrees with the results obtained in this study. The previous results showed that the plasmids carrying antibiotic resistance genes were not eliminated with EB. This could be due to high copy number of these plasmids in these isolates. However, these results are in agreement with that documented by Pallida et al. (1992) who demonstrated that the percent of cured plasmid DNA is not more than 20% in optimal conditions in *P. aeruginosa.*

### *Curing by elevated temperature*

Isolates of *P. aeruginosa* under study were consequently subjected to a plasmid curing procedure with elevated temperature, in order to cure their respective antibiotic resistance properties (Table 3). Elevated temperature (45°C) was used to cure the plasmid DNA that confers resistance to antibiotics in these isolates.

It is clear from Table 3 that the elevated temperature has a remarkable effect on all antibiotic resistance conferred by the bacterial isolates. All isolates lose their resistance to all antibiotics used in a range of 66-100%, 61-100%, 59-98%, 62-100%, 55-100% and 67-100% in P1, P6, P8, P10, P14 and P21 respectively.

From the obtained results, it can be concluded that curing by elevated temperature is an efficient curing agent among others. This may be due to the fact that the enzymes of DNA replication become more affected by this temperature. Our interpretation involves changing the shape (folding of the polypeptide) of the enzyme responsible for DNA replication of plasmids, though it may be that the change makes these enzymes inactive at this temperature. It was observed that inactivation of these enzymes may be due to the change in the folding of polypeptide at this temperature, that is, the enzymes are sensitive to elevated temperature (Kheder, 2002).

Studies have been made on the effect of elevated temperature on DNA synthesis and plasmid curing. Among them, May et al. (1964) obtained high frequency of loss after growing some bacterial strains at elevated temperature, and they observed that tetracycline and penicillinase positive strain of *S. aureus* grown at 43-44°C gave rise to increased proportion of tetracycline-sensitive and penicillinase negative bacteria (Al-Amir, 1999). More so, Ahmad (1989) and Bennett (2008) reported that there is a clear effect of elevated temperature on curing the plasmid DNA content from isolates of *P. aeruginosa* which is in agreement with the results of this study.

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