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ISOLATION OF A 29.5 KB PLASMID CONFERRING MUTIPLE DRUG RESISTANCE IN *PSEUDOMONAS AERUGINOSA*

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Abstract

Context: Worldwide emergence of plasmid mediated multi drug resistant bacterial strain is a growing concern, especially in hospital infections caused by *Pseudomonas aeruginosa*. Relation of plasmid and drug resistance in clinical isolates of *P. aeruginosa* by curing and transformation experiments is scanty..

Objectives: To isolate, purifyi and characterize plasmid DNA harbored in a selected *Pseudomonas aeruginosa* strain encoding multiple drug resistance and to perform transformation of the isolated plasmid into a sensitive strain of *Escherichia coli* LE 392 to judge transformation potential of the donor *P. aeruginosa* strain.

Materials and Methods: Plasmid DNA was isolated from a multidrug-resistant (MDR) strain of *P. aeruginosa* obtained from swab of a hospitalized burn patient by mini-scale method. DNA was purified, quantitatively estimated and electrophoresed on 0.8% agarose gel. Transformation was done as per Cohen and co-workers using plasmid DNA isolated from MDR *P. aeruginosa strain* as the donor and the *E. coli* LE 392 strain. The presence of plasmid in transformants checked through electrophoresis and the transformants was also tested for each drug resistance already recorded for the donor strain by disc diffusion method and again confirmed by spreading its culture on the selected antibiotic plate of different concentrations.

Results: A single plasmid of nearly 29.5 kb mass was isolated from MDR *P. aeruginosa* strain from clinical swab. This plasmid was transferred into sensitive and plasmid lacking recipient *E. coli* LE 392. Subsequent experiments on the transformed strain revealed that it acquired MDR and harbored a 29.5 kb plasmid which resembled to that of the donor strain proving that it encodes transferable MDR.

Conclusion: The MDR *P. aeruginosa* strain contained a transferable plasmid conferring resistance to ampicillin, chloramphenicol, cotrimoxazole, tetracycline and ciprofloxacin.

Key words: *Pseudomonas aeruginosa*; Multidrug –resistance, plasmid isolation, transformation.

Introduction

The worldwide emergence of multi-resistant bacterial strain is a growing concern, especially in hospital infections caused by *Pseudomonas aeruginosa*. Among nosocomial bacterial infections, those caused *P. aeruginosa* are associated with highest mortality rate, and are difficult to eradicate from infected tissues or blood because these microorganisms are virulent and have a limited susceptibility to antimicrobials (Kettner *et al.* 1995). Besides its innate resistance, acquired additional resistance due to plasmids is also a problem. Plasmid-mediated resistance involving modifying enzymes is particularly associated with topical antibiotic

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use and with sites where high levels of antibiotics are achieved antimicrobials (Kettner *et al.* 1995). There are several reports of serious outbreaks of multi drug-resistant (MDR) *P. aeruginosa* strains globally (Rossello *et al.* 1992, Sader *et al.* 1993, Bingen *et al.* 1996, Buttery *et al.* 1998). Plasmid- mediated resistance to various antimicrobial drugs has been demonstrated (WHO 2001, Govan 1996). The presence of MDR *Pseudomonas* strains in clinical isolates and in Bangladesh has been reported (Baqui and Rahman 1987, Asna and Haq 2000) with the transferable drug resistance capability especially to carbenicillin (Baqui and Rahman 1987). Rangneker *et al*. (1982) and Watanabe *et al.* (1983) have reported that gram-negative bacteria develop resistance to drug by acquiring plasmid and majority of these plasmid-mediated drug resistance are auto transferable to other organisms of the same or different species. Hossain and Rahman (1988) and Chowdhury *et al.* (1994) reported that *Pseudomonas* sp*.* isolated from cases of urinary tract infection in Bangladesh is resistant to commonly used antibiotics like ampicillin, tetracycline and co-trimoxazole.

An alarming increase in resistance of *Pseudomonas* spp. to various antimicrobial agents has been reported by many workers but studies demonstrating the relation of plasmid and drug resistance in clinical isolates of *P. aeruginosa* by curing and transformation experiments is scanty in our country. In the present study we have isolated a plasmid from MDR *P. aeruginosa* isolate, which was found responsible for resistance to ampicillin, chloramphenicol, cotrimoxazole, tetracycline and ciprofloxacin. Transformation of the isolated plasmid into a sensitive strain of *Escherichia. coli* LE 392 was also performed to judge transformation potential of the donor *P. aeruginosa* strain.

Materials and Methods

Isolation, purification and estimation of plasmid DNA: The present study was conducted between July 2007 and December 2008. The *P. aeruginosa* isolate (S_{14}) used was obtained from the pus culture isolated, characterized and identified from clinical cases in a previous prospective study (Begum 2004). Plasmid DNA was isolated from MDR strain (S_{14}) of *P. aeruginosa* by alkaline lysis method of Holmes and Quigley (1981). The DNA was purified with polyethylene glycol (PEG-8000) and quantitatively estimated by spectrophotometric method according to Sambrook *et al.* (1989). For quantitative estimation of Plasmid DNA, the absorbance of DNA was measured at 260 nm and 280 nm in an UV-Spectrophotometer (Shimadzu, Model UV-1200, JAPAN).

Characterization of the plasmid DNA by agarose gel electrophoresis: The plasmid DNA was subjected to electrophoresis on 0.8% agarose for 2 hr at 50 v using a horizontal slab gel electrophoresis apparatus (Mupid, Japan) according to the method of Meyers *et al.* (1976). The gel was stained for 2 h in 0.5 µg /ml of ethidium bromide solution and destained in water. A plasmid marker (λ- DNA digested with *Hind* III, Bangalore Genei, India) was used to calibrate the size of the isolated plasmid DNA. The gel was observed under an ultraviolet transilluminator (Model No.TM-15E, USA.) in a dark room. DNA bands in the gel were photographed using a Camera [Minolta α 7000 (Macro) Japan].

Transformation experiments: Transformation was done according to Cohen *et al.* (1972) using plasmid DNA isolated from MDR *P. aeruginosa strain* (S_{14}) as the donor and the *E. coli* strain LE 392, obtained from the Department of Molecular Biology and Biochemistry, Yamaguchi University, Japan, which was sensitive to all the previously tested drugs, as the recipient. The presence of plasmid in transformants was checked through electrophoresis and the transformants was also tested for each drug resistance already recorded for the donor strain.

Drug resistance test of transformants: Sixteen hour broth culture of the collected transformed strains (transformants) were grown at 37ºC, were spread on nutrient agar plates using sterilized glass spreader and allowed to dry. Then cotrimoxazole, ampicillin, chloramphenicol, gentamicin tetracycline and ciprofloxacin

discs were distributed on plates and kept at 4ºC for 4 h so that the antibiotic diffused on the agar media. The plates were then incubated at 37ºC for 16 h and the growth of the bacteria was observed. The presence of a clear zone around the disc was the index of sensitivity to the antibiotic. The absence of such a clear zone or the presence of some colonies within the clear zone indicated that the collected transformed strains were resistant to that antibiotic.

The drug resistant transformants tested by disc diffusion method were again confirmed by spreading its culture on the selected antibiotic plate of different concentrations. The plates were then incubated at 37ºC and observed on next day. The clear plate (no growth) indicated that the strains were sensitive to this selective concentration and presence of colonies on the plate indicated that the strains were resistant to that selective concentration. To characterize the multi-drug resistance whether it is plasmid mediated or not and to identify the responsive plasmid coding multiple drug resistance in selected *P. aeruginosa* strain, the plasmid DNA was isolated from the strain and transferred into sensitive *E. coli* LE 392, which does not have any plasmid of its own. Sensitivity of this strain was tested against at least six antibiotics like ampicillin, tetracycline, gentamicin, cotrimoxazole, chloramphenicol and ciprofloxacin prior to the transformation experiment and found completely sensitive to these drugs. Competent cells were prepared by calcium chloride procedure and transformation experiment was carried out by the method described by Cohen *et al.* (1972). After transformation experiment, growth on the control and experimental plates containing varying concentrations of ampicillin, tetracycline, gentamicin, cotrimoxazole chloramphenicol and ciprofloxacin was observed.

Results

Isolation, purification and estimation of plasmid DNA: It was observed that the ratio of optical density of the DNA solution at 260 nm and 280 nm was 1.78, which was very near to the standard value of 1.8 for pure DNA and hence the plasmid DNA isolated was almost pure. The plasmid DNA concentration obtained from 100 ml broth culture was roughly about 72μg. The purified plasmid DNA was subjected to slab gel electrophoresis on 0.8% agarose. Electrophoresis indicated the presence of a single plasmid and which was calculated to be approximately 29.5 kb in molecular mass as compared to the standard *Hind* III digested λ DNA marker (Fig.1).

Drug resistance study of the transformed strain: As presented in Table 1, growth on experimental plates containing ampicillin, cotrimoxazole, chloramphenicol and ciprofloxacin in the initial concentrations were observed but the growth decreased sequentially with the increase of antibiotic concentrations. On the other hand, no growth was observed on any of the control plates containing these antibiotics and sensitive *E. coli* LE 392 in same concentration. Colonies appearing on experimental plates were isolated and tested. *E. coli* LE 392 that was found sensitive to the antibiotics before transformation experiment became completely resistant to the above antibiotics except gentamicin, which remained sensitive after plasmid acquisition on Muller Hinton agar plate. These results strongly support the notion that drug resistance phenomenon in the selected MDR *P. aeruginosa* isolate was successfully transferred into sensitive *E. coli* LE 392 and makes it drug resistant. The results have been summarized in the Table 2.

Plasmid profile of transformed strain: Plasmid from the transformed strain was isolated and subjected to electrophoresis on 0.8% agarose. The electrophoresis profile of the transformed *E. coli* LE 392 showed that there was only a single plasmid DNA of molecular mass approximately 29.5 kb (Fig. 1). This plasmid DNA band was also found to be identical to that of the original strain, indicating that the transformation experiment was successful. These results confirmed the fact that the 29.5 kb plasmid harbored in the selected resistant *P. aeruginosa* strain carrying the gene (s) encoding multiple drug resistance and this drug resistance phenomenon was transferable in nature.

Table 1. Transformation of plasmid DNA isolated from *P. aeruginosa* into sensitive *E. coli* LE392

Plate made with antibiotic (μg/ml)	A30	A40	A60	S30	S40	S60	C30	C40	C60	T30	T40	T60	Ci30	Ci40	Ci60	G30	G40	G60
No. of transformed colonies appeared on the selection (experimental) plate	18	–	12	–	7	–	20	–	17	–	19	–	13	–	10	–	18	–

* No colonies grew in Control

A = Ampicillin, S = Cotrimoxazole, C = Chloramphenicol, T = Tetracycline, Ci= Ciprofloxacin and G =Gentamicin,

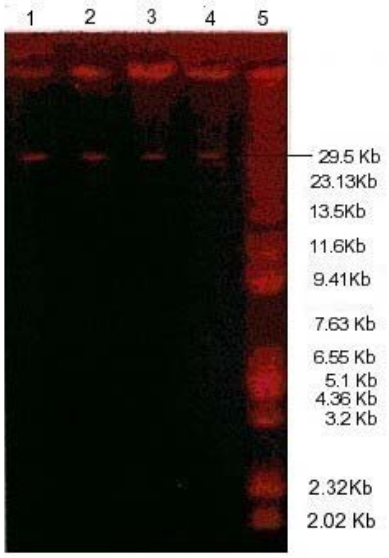

Fig. 1. Plasmid profile of isolated plasmid and transformed *E. coli* LE 392. Lane-1 and Lane-2.= Plasmid DNA from isolated multi drug resistant (MDR) *Pseudomonas aeruginosa.* Lane-3 and lane- 4= DNA of transformed *E. coli* LE 392 and Lane-5.= Marker DNA (λ DNA, *Hind* III digested).

Table 2. Antibiotic resistance of transformed *E. coli* LE392

Name of antibiotics	Concentration of antibiotic (μg/disc)	Diameter of clear zone (mm) on Muller Hinton agar plate
Cotrimoxazole	25	- (Resistant)
Ampicillin	10	- (Resistant)
Chloramphenicol	30	- (Resistant)
Tetracycline	30	- (Resistant)
Ciprofloxacin	5	- (Resistant)
Gentamicin	10	21 (Sensitive)

– no clear zone produced

Discussion

For a pure preparation of DNA, the ratio of optical density (O.D) at 260 nm and 280 nm (OD_{260}/OD_{280}) should be 1.8. The absorbance ratio (OD_{260} / OD_{280}) of the isolated plasmid DNA from multidrug -resistant (MDR) *P. aeruginosa* strain in the present study was estimated to be 1.78, indicating that it was almost pure. The electrophoretic pattern showed that this *P. aeruginosa* strain contained a single plasmid calculated to be with approximate molecular mass of 29.5 kb. Plasmid harboring in *P. aeruginosa* is not new. Vazquez *et al* (1992) reported that about 21.4% of their nosocomial *P. aeruginosa* isolates contained between one to six plasmids with molecular sizes ranging from 1 to 80 MDa.

Plasmid DNA was isolated from the transformed *E. coli* LE 392 and when electrophorized with the reference to that of original strain, the plasmid profile of transformed bacteria showed the presence of plasmid of approximately 29.5 kb molecular mass which was identical to the original *P. aeruginosa* plasmid indicating

successful transformation. After transformation experiment, the transformed strain appearing on plates were tested for their resistance to cotrimoxazole, ampicillin, chloramphenicol, tetracycline, gentamicin and ciprofloxacin by disc diffusion method. It was observed that *E. coli* LE 392 that was sensitive to these antibiotics before transformation became completely resistant to cotrimoxazole, ampicillin, chloramphenicol, Tetracycline and Ciprofloxacin due to this plasmid acquisition. However, the activity of gentamicin remained unchanged that is it was sensitive even after plasmid acquisition. This transformed strain was again tested by spread plate method using cotrimoxazole, ampicillin, chloramphenicol, tetracycline and ciprofloxacin on which several colonies were appeared, but no drug resistant colony was on the control plates. The study indicated that these drug resistances were transferred from *Pseudomonas aeruginosa* into *E. coli* LE 392.

Conclusion

The findings suggested that plasmid of molecular mass of approximately 29.5 kb in size was transferred and resistance property of transformed *E. coli* LE 392 to cotrimoxazole, ampicillin, chloramphenicol, tetracycline and ciprofloxacin was due to the presence of that extrachromosomal DNA or plasmid. The result also suggested that the resistance property towards gentamicin in strain S_{14} might not be plasmid mediated, since no property was found in case of transformed *E. coli* LE 392.

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