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SEARCH FOR DRUG AND METAL RESISTANCE PLASMIDS FROM INDIGENOUS CLINICAL PSEUDOMONADS

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Abstract

On the bases of morphology, cultural and biochemical characteristics, 50 indigenous clinical pseudomonads were identified as *Pseudomonas aeruginosa*. Resistance patterns of antibiotics upto 500 μg mL^{-1} and inorganic metallic ions upto 3 mg mL^{-1} were determined by medium incorporation method. Five of the isolates lost their resistance to erythromycin after curing for plasmid elimination with 5 mg mL^{-1} of acridine orange, thereby indicating the extrachromosomal location of the resistance determinants. Agarose gel electrophoresis using `Mini prep' method for plasmid isolation revealed that six isolates harbour plasmids of different molecular weights (5-28 kb) with Hind III digest of λ DNA as ladder. Some of the pseudomonad isolates were able to transfer their resistance to *Escherichia coli* BU 40 by *in vivo* gene transfer plasmid conjugation.

Introduction

The emergence of drug resistance has been regarded as an inevitable genetic response to the selective pressure imposed by antimicrobial therapy (Lyon & Skurray, 1987). Appearance of antibiotic resistant strains was confirmed by the discovery of gene transfer and the demonstration that bacteria can acquire additional genetic material in the form of extrachromosomal plasmid DNA (Broda, 1979). The R-factors (plasmids) may also contain genes for other characteristics e.g., some strains of *Escherichia coli* and *Salmonella* have been shown to mediate resistance to mercury, cobalt and nickel (William, 1978). A plasmid was found responsible for resistance in *Pseudomonas aeruginosa* to the compounds of tellurium, mercury (Clark *et al.*, 1977), boron and chromium (Summers & Silver, 1978). The plasmids can be cured from the bacterial cells spontaneously or with dyes such as acridine orange and elevated sub-lethal temperatures (Van der Vossen *et al.*, 1994). The curability has been used to ascertain plasmid associated nature of genes (Salisbury *et al.*, 1972). Some R factors can be transferred to a number of distant genera and are referred as broad-host range plasmids. The present study was undertaken to determine the drug and metal resistance patterns of the indigenous clinical pseudomonads and to isolate and characterize the R-plasmids by agarose gel electrophoresis. Plasmid conjugation was also performed in order to check the transferability of the plasmid-borne resistance determinants.

Material and Methods

Bacterial Isolates: Fifty clinical isolates of Pseudomonads were collected from various pathological laboratories and hospitals of Karachi.

Antibiotics and metal salts: Eight commonly used drugs and 8 different metal salts were used. Erythromycin and chloramphenicol were dissolved in absolute ethanol, trimethoprim and lead acetate in DMSO (Merck) while mercuric chloride was dissolved in a few drops of ethanol and the volume was made up with distilled water. All other antibiotics and metal salts were dissolved in distilled water. Stock solutions of 10 mg mL^{-1} were prepared, sterilized by 0.45 μm millipore filters and refrigerated.

Media: Nutrient and MacConkey's agar (Oxoid) were used for identification and screening the cultures for drug and metal resistance. Luria basal agar (LBA) was used for curing and conjugation experiments. Cultures were maintained in Caryblair maintenance medium (Oxoid) at 4°C. Glucose, maltose and lactose (Merck) were used for carbohydrate utilizing ability.

Chemicals: Acridine orange, ethidium bromide [Sigma (10 mg and 50 mg mL^{-1} stock solutions respectively), agarose, TAE buffer, Tris, EDTA, boric acid, lysozyme (Sigma), bromophenol blue (Aldrich), NaOH, ethanol (Merck), glycerol, SDS and oxidase reagent (Sigma) were used.

DNA marker: Hind III digest of λ DNA (Sigma) was used as ladder (Hind III generates 8 fragments of λ DNA).

Drug and metal resistance: Isolates were subjected to antibiotic and metal resistance screening by replica plating (Lederberg *et al.*, 1952). The highest concentration of an antibiotic or metal salt showing growth of all the replicated clones was taken as the resistance level of the strain for that particular drug or metal.

Plasmid Curing: In order to determine the location (plasmid or chromosomal) of the drug and metal resistance determinants, the curing experiments were performed

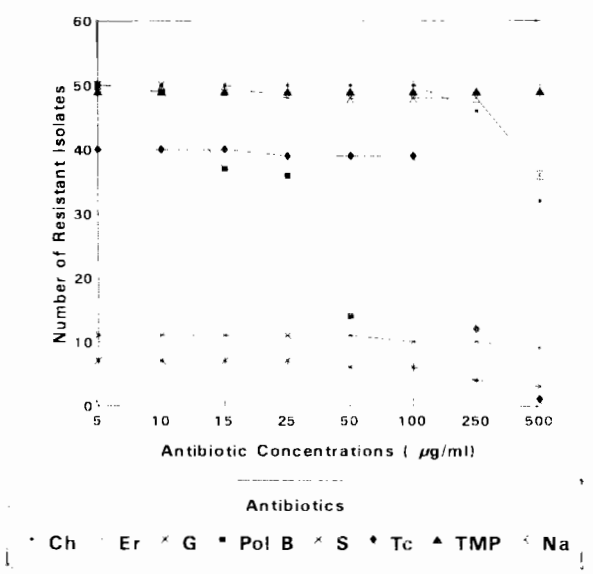

Fig.1. Occurence of antibiotic resistance in the isolates of *Pseudomonas aeruginosa* at various concentrations.

using physical (high temperature i.e., 44°C) (Novick, 1990) and chemical agents (acridine orange and ethidium bromide (Van der Vossen *et al.*, 1994).

Plasmid Conjugation: Plasmid conjugation was performed as per Miller (1972) and Forbes & Schaberg (1983) for the transfer of drug and metal resistance determinants from three representative pseudomonads (used as donors) to *E. coli* BU 40 (used as the recipient).

Isolation of Plasmid DNA: For the isolation of plasmid DNA, the `miniprep' method of Zhou *et al.*, (1990) was followed. The samples were then subjected to agarose gel electrophoresis and photographed over UV transilluminator by high speed Kodak film (UV Products, UK).

Results and Discussion

Multiple drug resistance against commonly used antibiotics (by plate incorporation) was found very common in all the isolates. Surprisingly, all the isolates showed resistance. The resistance to chloramphenicol, erythromycin, trimethoprim and nalidixic acid was the most common (Fig.1). Resistance to gentamicin (an aminoglycoside) was low as also reported earlier by Khursheed & Khatoon (1984). A number of antibiotic preparations employed for human and animal use are contaminated with chromosomal DNA of the antibiotic-producing organisms. The uptake of this DNA by bacteria and its functional incorporation into bacterial replicons would lead to the generation of antibiotic resistance determinants (Chakrabarty *et al.*, 1990; Webb & Davies, 1993).

Resistance against the metal salts was observed in all the isolates. Almost all the isolates showed resistance to the metal salts particularly against zinc sulphate (upto 3000 $\mu g\ mL^{-1}$). (Fig 2). The toxicity of zinc and cadmium is reduced by an operon like system in *E. coli* and *S. aureus*. Mercuric chloride however, has been found to be the most toxic (since only 12% of the isolates survived upto 50 $\mu g\ mL^{-1}$). Survival of bacteria at higher concentrations of metallic salts could be due to genetic adaptation in the extreme environments (Silver *et al.*, 1978). Presumably resistant bacteria convert more toxic forms to less toxic forms through enzymatic activities (Robinson & Tuovinen, 1984).

Chemical-mediated curing experiments were conducted to ascertain the location of the antibiotic and metal resistance determinants. The inhibition of the isolates could not be observed upto 4,000 $\mu g\ mL^{-1}$ of acridine orange. Ethidium bromide (4 $mg\ mL^{-1}$) and high temperature (44°C) - mediated curings was also tried but without significant success. After 5,000 $\mu g\ mL^{-1}$ of acridine orange treatment, the erythromycin resistance was lost in 5 isolates only. On the other hand, metal resistance could not be cured at all under the similar conditions. It has earlier been reported that the susceptibility to curing agents varies among plasmids whereby, some are not curable by ethidium bromide or acridine orange (Khatoon, 1971).

Agarose gel electrophoresis revealed the presence of plasmids of different size ranging from 5-28 kb in 6 isolates. Only 17% isolates showed the presence of resolvable plasmids while 10% showed curable plasmids for erythromycin resistance determinant (Fig.3) alongwith the Hind III digest of λ DNA used as marker (only first two bands are visible in the photograph).

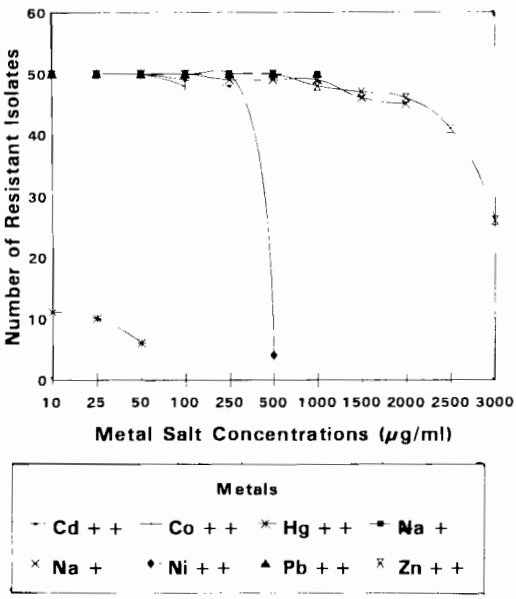

Fig.2. Occurence of metal resistance in the isolates of *Pseudomonas aeruginosa* at various concentrations.

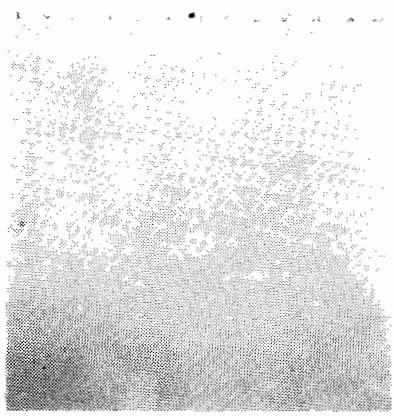

Fig.3. Gel electrophoresis showing plasmid profile of the uncured (normal) and cured clones of some isolates of *Pseudomonas aeruginosa* using 0.8% agarose.

1. Hind III λ digest (marker)
2. PA-29 (10 kb)
3. Cured of PA-29
4. PA-06 (10 kb & 8 Kb)
5. Cured of PA-06
6. Cured of PA-35
7. PA-35 (12 kb)
8. Cured of PA-02
9. PA-02 (28 kb)
10. PA-05 (16 kb, 5 kb)
11. Cured of PA-05
12. PA-22 (16 kb)

Using tube and millipore filter mating methods (Forbes & Schaberg, 1983) erythromycin resistance gene of *Pseudomonas aeruginosa* was successfully transferred to the recipient (*E. coli* BU 40). Rasool & Khan (1993) reported the transfer of antibiotic and bacteriocinogenic genes from *Pseudomonas* to *E. coli*. Thus, in most of the isolates, drug and metal resistance determinants are located on the chromosome although erythromycin resistance marker in 10% cases is plasmid borne. Richmond (1979) also stressed that all antibiotic resistance genes are not plasmid-oriented. The loss of erythromycin resistance in only 10% of the isolates, in one set of the experiments, could be regarded as the plasmid-borne location of erythromycin determinant. Failure in plasmid curing in another set of experiments with the same isolates may indicate that this resistance determinant may be transposed to the chromosome (Bukhari *et al.*, 1977).

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