Punjab Univ. J. Zool., Vol. 12, pp. 43-48, 1997

PLASMID-MEDIATED ANTIBIOTIC RESISTANCE IN CLINICAL ISOLATES OF *PSEUDOMONAS AERUGINOSA*

BASHARAT AHMED AND A.R. SHAKOORI

Department of Zoology, University of Azad Jammu & Kashmir, Muzaffarabad (BA) and Department of Zoology, University of the Punjab, Lahore 54590 (ARS), Pakistan

Abstract: A total of eighteen *Pseudomonas aeruginosa* isolated from CMH Muzaffarabad, were screened for their resistance to the antibiotics, Ampicillin, Chloramphenicol, Doxycycline, Erythromycin, Gentamicin, Kanamycin, Minocycline, Nalidixic acid, Penicillin, Streptomycin, Urixin and Velosef. Generally, the isolates showed the highest frequency of resistance to Penicillin and lowest to Velosef, followed by Doxycycline and Erythromycin. The resistant isolates showed eight patterns of antibiotics resistance at a level as high as 1000 μg/ml. Some of the antibiotic determinants were cured by acridine orange treatment, indicating the widespread antibiotic resistance is mediated through plasmids among *Pseudomonas aeruginosa*.

Key words: Antibiotic resistance, plasmid, bacterial isolates.

INTRODUCTION

Pseudomonas aeruginosa is notorious for its resistance to most commonly used antimicrobial agents. It appears to be a common hospital pathogen because of its resistance to a number of antibiotics, including, Carbenicillin, Gentamicin, Tobramycin (Tsakris *et al.*, 1992). It appears that widespread use of such drugs has resulted in the development of a variety of R-factors which can inactivate these antibiotics by phosphorylation, adenylation, acetylation or by a combination of them (Erova *et al.*, 1989; Eleanor and Sallch, 1992). Montelli *et al.* (1989) showed the highest level of resistance against 23 antimicrobial agents in *Pseudomonas* strains. Moreover, the authors also found a significant increase in resistance to some aminoglycosides and beta-lactam compounds. *P. aeruginosa* have also shown resistance to extended spectrum cephalosporins (Patrice *et al.*, 1993).

Evidences have been provided from several countries about the misuse and abuse of antibiotics and this indiscriminate use of these drugs is contributing to a world-wide increase in resistant strains of bacteria (Schneider and Schweisfurth, 1991). Widespread use of these antibiotics has failed to eradicate infections and instead an increase in antibiotic resistant bacteria have been observed all over the world (Maple *et al.*, 1989; Nazarov *et al.*, 1989; Dakshinkar *et al.*, 1992; Fung-Tome *et al.*, 1993).

0079-8045/97/0001-0041 $ 03.00/0

The present study deals with high levels of multiple antibiotic resistance of *P. aeruginosa* and their plasmid associated nature (Tsakris *et al.*, 1992; Blahova *et al.*, 1992; Padilla and Vasquez, 1993). R-plasmids like other extrachromosomal elements, can be lost spontaneously from the host cell because of some errors in replication or segregation (Watanabe and Lyang, 1962b). These losses (elimination or curing) can be increased by treating the host cell with acridine orange (Watanabe and Lyang, 1962a). Thus plasmid loss or curing can be used as a criterion for determining plasmid associated nature of antibiotic resistance.

In Pakistan, like other developing countries there is a general increase in antibiotic resistance especially to all commonly used antibiotics (Khursheed and Khatoon, 1984; Naqvi *et al.*, 1990; Ansari and Khatoon, 1994). Hence there is a need to monitor the prevailing levels of resistance so that an effective system for bacterial therapy can be developed.

MATERIALS AND METHODS

Pseudomonas aeruginosa strains were isolated from patients at Combined Military Hospital (CMH) Muzaffarabad. Bacterial cultures were maintained in freezing glycerol nutrient media at -20°C. For routine experiments, the cultures were maintained on nutrient agar plates at 4°C and subcultured bimonthly. Difco (USA) nutrient broth and agar were used for the screening of cultures for antibiotic resistance.

Antibiotics

Twelve antibiotics were used in these studies. These were, Ampicillin (A), Chloramphenicol (C), Doxycycline (D), Erythromycin (E), Gentamicin (G), Kanamycin (K), Minocycline (M), Nalidixic acid (N), Penicillin (P), Streptomycin (S), Urixin (U) and Velosef (V). A stock solution (10 mg/ml) of each antibiotic was made in distilled water. Chloramphenicol was dissolved in ethanol. These stock solutions were used for calculating the dose of each antibiotic used in resistance experiments. All solutions were sterilized by millipore (0.45 mμ) filters and refrigerated.

Antibiotics susceptibility testing

The cultures obtained were further identified on the basis of pigment production. The pure isolates were subjected to antibiotic resistance screening by replica plate method (Lederberg and Lederberg, 1952). For this purpose, a broth culture of the test strain was plated on nutrient agar plate to obtain isolated colonies. A few colonies were picked on to a master plate, incubated overnight and replicated on nutrient agar plates containing different concentrations of all the antibiotics. The highest concentration of an antibiotic showing growth of all the replicated clones was taken as the resistant level of the strain for the particular antibiotic.

Acridine orange mediated plasmid curing

The method of Hirota (1960) was followed for this purpose. A small inoculum ($2x10^{-2}$ - $5x10^{-2}$ bacteria) was added to varying concentrations of acridine orange broth and incubated at 37°C overnight. Cultures containing the highest concentration of acridine orange in which growth was clearly visible were diluted and spread on nutrient agar plates containing appropriate antibiotic.

RESULTS

The antibiotic resistance was screened against eighteen clinical isolates of *P. aeruginosa*. Overall 86% of strains were resistant to Penicillin (P), 80% to Kanamycin (K), 76% to Gentamicin (G), 75% to Ampicillin (A), 73% to Streptomycin (S) and Urixin (U), 67% to Chloramphenicol (C) and Doxycycline (D), 66% to Minocycline (M), 65% to Velosef (V), 63% to Nalidixic acid (N) and 60% to Erythromycin (E).

The results of MICs against twelve antibiotics resistance were obtained and indicated in a comparative account of antibiotics resistance of isolates at differnt levels in Table 1. Generally, the isolates showed the highest frequency of resistance to Penicillin (55%) and the lowest to Velosef (5%) followed by Doxycycline (11%) at 1000 μg/ml (Table I). There was a slight decrease in the number of Penicillin resistant isolates at the levels of 250-1000 μg/ml compared with the level of 25 μg/ml. At 500 μg/ml level the isolates showed a considerable decrease in the resistance frequency of almost all the antibiotics tested.

Table 1: Occurrence of antibiotic resistance of *P. aeruginosa* at six different concentrations.

Antibiotics	Number of resistant cultures at concentrations of (μg/ml)					
	25	50	100	250	500	1000
Ampicillin	17	17	17	16	8	6
Chloramphenicol	17	16	15	14	6	5
Doxycycline	18	17	16	13	7	2
Erythromycin	16	14	13	11	8	3
Gentamicin	18	18	17	14	10	5
Kanamycin	18	18	18	17	9	7
Minocycline	17	16	15	12	7	4
Nalidixic acid	16	15	14	13	6	4
Penicillin	18	18	18	17	12	10
Streptomycin	18	18	17	13	9	5
Urixin	17	17	16	13	9	7
Velosef	16	16	15	14	8	1

Multiple antibiotic resistance among *P. aeruginosa* isolated from clinical sources is not uncommon. The strains screened were 88% resistant to three or more of the antibiotics at 100 μg/ml, 78% were resistant to three or more antibiotics at 250 μg/ml, 44% were resistant to three or more antibiotics at 500 μg/ml and 28% were resistant to one or more antibiotics at 1000 μg/ml. The resistant cultures showed eight differnt patterns of antibiotics resistance at a level as high as 1000 μg/ml (Table II). Among these, AKPU resistance was found to be the most common.

Table II: Antibiotic resistance pattern at 1000 μg/ml in *P. aeruginosa* isolated from different clinical specimens.

Pattern Number	Resistance pattern	No. of cultures
1	ACDEGKMNPSV	1
2	CEGKMNPSU	1
3	CEGKMPSU	1
4	ACGMPSU	1
5	ACDGNP	1
6	AKPU	3
7	CNPU	1
8	KPU	1

Abbreviations used: Ampicillin, A; Chloramphenicol, C; Doxycycline, D; Erythromycin, E; Gentamycin, G; Kanamycin, K; Minocycline, M; Nalidixic acid, N; Penicillin, P; Streptomycin, S; Urixin, U; Velosef, V.

Loss of antibiotic resistance after plasmid curing

Two representative multiple antibiotic resistant *P. aeruginosa* cultures (patterns 1 and 2) were selected for plasmid curing. Out of 100 colonies each from two treated cultures some had lost the resistance to one or the other antibiotics. However, a total loss to Velosef was found in one of the cultures.

DISCUSSION

The results indicate that antibiotic resistance among indigenous clinical samples of *P. aeruginosa* is very common. Of the strains screened for resistance, 44% were resistant to three or more antibiotics at 500 μg/ml and 28% were resistant to three or more antibiotics at 1000 μg/ml. These findings are similar to those reported by earlier workers (Eleanar and Sallch, 1992; Tsakris *et al.*, 1992; Fung-Tome *et al.*, 1993; Patrice *et al.*, 1993). The resistance to doses as high as 1000 μg/ml is alarming because if bacteria become resistant to such high levels of antibiotic, disease treatment with antibiotics would become quite difficult and may not be possible.

A total resistance towards Penicillin was exhibited by *P. aeruginosa* (Dakshinkar *et*

al., 1992). In this study the highest frequency of resistance was against Velosef at 1000 μg/ml and it seems to be the most effective antibiotic for treating infections of *P. aeruginosa* with high doses. The other effective antibiotics at 1000 μg/ml were Doxycycline and Erythromycin.

In the present study the maximum number of the resistant cultures were found at the level of 25 μg/ml (95%). Only 4.6% of the cultures were sensitive to all the twelve antibiotic tested. We also observed very high resistance levels in 18 strains for which MICs of the antibiotics were tested. The multiple antibiotic resistance may result from the selection of resistant mutants through the widespread use of antibiotics.

In view of the overall high incidence of multiple antibiotic resistance among *P. aeruginosa*, the possibility of the presence of R-plasmids was explored. The loss of resistance to single or multiple antibiotics after acridine orange treatment of cultures points to the fact that observed antibiotic resistance was plasmid borne. Neither of the two cultures showed a loss of resistance to all the antibiotics simultaneously. This shows that different plasmids determine the resistance against different antibiotics. Plasmid analysis undertaken by Tsakris *et al.* (1992) showed that *P. aeruginosa* harbour plasmids ranging in size from 20 to 100 Mda and the larger plasmids (100 Mda) encoded high level resistance to Gentamiccin and Tobramycin, as was also seen to Kanamycin and Streptomycin by Blahova *et al.* (1992) and to Ampicillin and Chloramphenicol by Padilla and Vasquez (1993).

Loss of Velosef resistance in all the colonies of two of the cured cultures could be due to the fact that the corresponding plasmid may be very small and fragile which could not resist the curing treatment in all the cells. Low percentage of curing in other resistant determinants was observed in these studies. Analogous results have been reported in *Aerobacter aerogenes* by Khatoon and Mohammad (1986) and in *Staphylococci* by Rasool *et al.* (1987). Since curing was not observed in all the resistant determinants it may be presumed that some plasmids were present in integrated state and hence stable as has been suggested by Hirota (1960).

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(Received: May 22, 1997)