TRANSFER OF PLASMID-MEDIATED ANTIBIOTIC RESISTANCE IN STRAINS OF *PSEUDOMONAS AERUGINOSA* ISOLATED IN AUCKLAND

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PSEUDOMONAS AERUGINOSA causes severe infections, particularly in debilitated or immunosuppressed patients who are difficult to treat because the organism is resistant to many antibiotics. Gentamicin has been the most effective antibiotic for pseudomonas infections and initially the majority of strains were sensitive to it. However, recent reports show that the incidence of gentamicin-resistant strains of *P. aeruginosa* is increasing (Meyer *et al.*, 1976), that this resistance is frequently plasmid-borne (Witchitz and Chabbert, 1971; Knothe, Krcmery and Stietzen, 1973; Bryan, Shahrabadi and Van den Elzen, 1974; Jacoby, 1974a) and that it is often associated with resistance to other aminoglycoside antibiotics.

This paper describes the results of a survey of the antibiotic-resistance patterns of clinical isolates of *P. aeruginosa* in Auckland and their ability to transfer this resistance by conjugation.

MATERIALS AND METHODS

Organisms. All strains of *P. aeruginosa* isolated at Auckland Hospital from clinical specimens during 1975 were examined for their susceptibility to gentamicin and all resistant strains were included in the investigation. The characteristics and source of the recipient strains used in tests for conjugal transfer of antibiotic resistance are shown in table I.

Culture media. Trypticase Soy Broth (Difco) and trypticase soy broth solidified with 1.2% New Zealand Agar (Davis Gelatine Ltd) were used for most experiments. Minimal agar was that specified by Clowes and Hayes (1968) with Difco agar used to solidify the minimal salts solution. MacConkey agar was Oxoid Code No. CM3 agar. Selective plates for genetically distinct strains were prepared from minimal agar supplemented with the appropriate amino acids at a final concentration of 20 µg/ml for the growth requirements of the selected strain.

Antibiotics. Freshly prepared solutions of antibiotics were added to the selective media to give the following final concentrations (µg/ml) rifamycin 50, gentamicin 5, tobramycin 5, amikacin 25.

Determination of antibiotic resistance. Antibiotic susceptibility was determined by the Stokes method (Stokes, 1968) on Diagnostic Sensitivity Test Agar (DST, Oxoid). The control organism was *P. aeruginosa* strain PsAH1 isolated in this laboratory and susceptible to gentamicin (MIC 0.8 µg/ml), tobramycin (MIC 1.6 µg/ml), amikacin (MIC 2.0 µg/ml), carbenicillin (MIC 25 µg/ml). The discs used were gentamicin 10 µg and tobramycin 10 µg (Eli Lilly), amikacin 10 µg (Bristol Meyer), carbenicillin 100 µg (Beecham Research Laboratories), and sisomicin 10 µg

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(Schering). Minimum inhibitory concentrations (MICs) were determined by an agar-dilution technique in DST agar with the following ranges of antibiotic concentrations in doubling dilutions: gentamicin and tobramycin 160–0·31 µg/ml, amikacin 400–0·4 µg/ml, sisomicin 50–0·4 µg/ml, kanamycin 50–0·1 µg/ml, streptomycin 50–0·1 µg/ml, carbenicillin 200–32 µg/ml. The plates were spot inoculated with a Clement's antibiotic replicator and the inoculum was $c. 3 \times 10^4$ organisms per spot. Organisms were classified as resistant if the MIC of gentamicin, tobramycin or sisomicin was ≥ 5 µg/ml, of amikacin, kanamycin or streptomycin ≥ 25 µg/ml, or carbenicillin ≥ 125 µg/ml. *E. coli* strain NCTC9033 was the control for these tests.

Transfer of antibiotic-resistance determinants by conjugation. Broth cultures of donor and recipient strains were grown overnight and 0·1 ml of donor culture plus 1 ml of recipient culture were added to 5 ml of fresh trypticase soy broth. After overnight incubation at 37°C, 0·1-ml volumes of the mixed culture were plated on to a series of MacConkey agar plates that contained rifamycin plus each antibiotic to which the donor was resistant separately. The colonies that grew on these plates were purified by restreaking on MacConkey agar, and 10 colonies from each selective plate were identified by growth on minimal agar, with and without supplements, and by biochemical tests, and tested for antibiotic sensitivity with Multidisks (Alpha Biologicals Ltd). The donor and recipient strains were also plated on MacConkey agar that contained only rifamycin or each antibiotic to which the recipient was resistant as controls.

Pyocine typing and serotyping. Pyocine typing and serotyping of the donor strains was performed by the National Health Institute, Wellington, NZ by the technique of Gillies and Govan (1966) for pyocine typing and the method of Habs (1957) for serotyping.

TABLE I
Characteristics of recipient strains

Strain	Phenotype*	Source
<i>Pseudomonas aeruginosa</i> PsAH1-1	Wild strain rif ^R	This laboratory
<i>Pseudomonas aeruginosa</i> OT2-1	rif ^R leu ⁻	Dr J. S. Loutit University of Otago, Dunedin
<i>Pseudomonas aeruginosa</i> OT47-1	rif ^R ilv. leu ⁻	
<i>Escherichia coli</i> K 12 J53-2	rif ^R met ⁻ pro ⁻	Dr N. Datta Royal Postgraduate Medical School, Hammersmith Hospital, London

* rif^R = rifampicin resistant.

leu⁻ = leucine

ilv. = isoleucine, valine

met⁻ = methionine

pro⁻ = proline

} amino-acid requirements.

RESULTS

Antibiotic-resistance patterns of P. aeruginosa isolates

Of the 422 isolates of *P. aeruginosa* examined, 36 (8·5%) from 23 different sources were resistant to gentamicin. Details of the source and type of the 23 gentamicin-resistant strains that were investigated further are shown in table II. All except one strain (PsAH9) were isolated from patients. Seven strains

TABLE II
A summary of the site, primary diagnosis, antibiotic resistance and type of the gentamicin-resistant strains of P. aeruginosa

Strain no.	Site	Primary diagnosis	Service	Resistant to*							Pyocine type	Serotype
				Gm	Tm	Ak	Cb	Ss	Nm	Sm		
PsAH3	Groin	Leukaemia	Haematology	+	+	-	-	+	+	-	17	11
PsAH4	Groin	Leukaemia	Haematology	+	+	-	-	+	+	+	13	9/13
PsAH5	Urine	Acute renal failure	Critical care	+	-	-	+	-	+	+	misc	6/9
PsAH6	Groin	Leukaemia	Haematology	+	+	-	-	+	+	+	17	11
PsAH7	Sputum	Leukaemia	Haematology	+	+	-	-	+	+	+	17	11
PsAH8	Skin ulcer	Infected skin ulcer	Gen. medicine	+	+	+	-	+	+	+	10	8
PsAH9	Sink, sluice room											
PsAH10	Urine	Haematology ward	Haematology	+	+	-	+	+	+	+	13	10
PsAH11	Groin	Multiple fractures	Urology	+	+	-	+	+	-	+	1	11
PsAH12	Groin	Leukaemia	Haematology	+	+	-	-	+	+	+	17	11
PsAH13	Groin	Leukaemia	Haematology	+	+	-	-	-	+	+	misc	6
PsAH14	Skin	Peptic ulcer	Gen. surgery	+	+	-	-	+	+	+	misc	11
PsAH15	Urine	Alcoholism, UTI	Gen. medicine	+	-	-	-	-	-	-	5	3
PsAH16	Bronchial washings											
PsAH17	Bronchial washings	Lung abscess	Gen. medicine	+	+	-	-	+	+	+	misc	11
		Renal transplant, resp. failure										
PsAH18	Sputum	Lymphoma	Renal	+	+	-	-	+	+	+	misc	11
PsAH19	Urine	UTI	Haematology	+	+	-	+	+	+	+	misc	11
PsAH20	Urine	Subarachnoid haem, UTI	Gen. surgery	+	+	-	+	+	+	+	1	10
PsAH21	Urine	Cholangitis	Gen. medicine	+	+	-	-	+	+	+	10	11
PsAH22	Wound	Ulcerative colitis	Gen. surgery	+	+	-	-	+	+	+	17	9
PsAH23	Urine	Renal transplant	Gen. surgery	+	+	-	+	+	+	+	1	11
PsAH24	Urine	UTI	Renal	+	-	-	+	+	+	+	17	6
PsAH25	Urine	Renal transplant, UTI	Gen. medicine	+	+	-	-	-	-	-	17	11
		Renal transplant, UTI										
PsAH26	Urine	Renal transplant, UTI	Renal	+	-	-	+	+	+	+	1	9
		Critical care										
				+	+	-	+	+	+	+	1	9

* Gm = gentamicin; Tm = tobramycin; Ak = amikacin; Cb = carbenicillin; Ss = sisomicin; Nm = neomycin; Sm = streptomycin; Km = kanamycin; UTI = urinary-tract infection.

were isolated from patients with leukaemia but only one patient, who died from a pseudomonas septicaemia, had a clinical infection; the remainder were isolated from routine groin swabs. Ten strains were isolated from patients with urinary-tract infection; three patients had renal transplants and four had other underlying diseases. Three strains (PsAH16, 17 and 18) were isolated from bronchial washings collected from three patients at bronchoscopy. These strains had the same pyocine type and serotype; subsequent investigations showed that strain PsAH18 was present in the suction tubing used to obtain the bronchial washings from all three patients. The metal connections between the suction tubing and specimen bottles had not been adequately cleaned and were contaminated with this strain, which was present in the sputum of one patient. This isolate was initially resistant to carbenicillin but the strains isolated at bronchoscopy from the other two patients were sensitive when tested later.

The results of antibiotic-sensitivity tests on the 23 strains are shown in table III. For six (26%) strains the MIC of gentamicin was $\leq 10 \mu\text{g/ml}$; for five of these the MIC of tobramycin was $< 5 \mu\text{g/ml}$ and they could be treated with tobramycin. Only one strain (PsAH8), isolated from a skin ulcer, was resistant to amikacin, gentamicin and tobramycin. Sixteen (70%) strains were highly resistant to gentamicin ($\text{MIC} > 40 \mu\text{g/ml}$) and for one strain the MIC was $20 \mu\text{g/ml}$. Four strains were sensitive to kanamycin ($\text{MIC} < 10 \mu\text{g/ml}$) and four strains were sensitive to sisomycin. No strain was sensitive to streptomycin.

TABLE III
Antibiotic sensitivity of gentamicin-resistant strains of P. aeruginosa

Number of strains	MIC ($\mu\text{g/ml}$) of*					
	Gm	Tm	Ak	Ss	Km	Cb
10	> 160	> 160	< 25	> 25	> 25	< 125
2	> 160	> 160	< 25	> 25	< 25	< 125
4	> 160	> 160	< 25	> 25	> 25	> 125
2	10	< 5	< 25	< 10	> 25	> 125
1	10	10	< 25	10	20	500
1	20	5	50	20	> 25	< 125
2	10	< 5	< 25	< 10	< 25	< 125
1	10	< 5	< 25	< 5	10	> 125

* Footnotes as in table II.

Transfer of resistance determinants

None of the 23 gentamicin-resistant strains of *P. aeruginosa* transferred resistance to *E. coli* K12 strain J53-2. Twenty strains (87%) transferred all or some of their resistance determinants to recipient *P. aeruginosa* strains OT2-1

and OT47-1. There was no difference in the transfer of determinants to either recipient strain.

Table IV shows the pattern of resistance determinants transferred by the clinical isolates. For the three strains that failed to transfer gentamicin resistance, the MIC of gentamicin was low ($\leq 10 \mu\text{g/ml}$). Strains for which the MIC of gentamicin and tobramycin was high transferred resistance determinants to both recipient strains. Seven strains were resistant to carbenicillin in addition to gentamicin and tobramycin or both, but only three strains transferred carbenicillin resistance. One strain (PsAH8) was resistant to gentamicin, tobramycin and amikacin and although the MICs of gentamicin ($20 \mu\text{g/ml}$) and tobramycin ($5 \mu\text{g/ml}$) were not high, all three resistance determinants were transferred to the recipient strains of *P. aeruginosa*.

Serotyping and pyocine typing of the 23 gentamicin-resistant strains (table II) yielded several patterns and the largest number of isolates with the same pattern was five; 10 strains had unique patterns. Of the five strains that gave the same pattern, four were isolated in one ward, suggesting cross infection.

TABLE IV
Patterns of transfer of resistance determinants from gentamicin-resistant P. aeruginosa

Pattern of resistance determinants* in wild strain	Number of wild strains with the given pattern	Pattern of resistance determinants transferred	Number of strains that transferred the given pattern
Gm Tm	12	Gm Tm	12
Gm Cb	2	Gm	2
Gm Tm Ak	1	Gm Tm Ak	1
Gm Tm Cb	5	Gm Tm Cb	3
		Gm Tm	2
Gm	3	Not transferred	...

* Footnotes as in table II.

DISCUSSION

This study shows a significant incidence of gentamicin resistance in strains of *P. aeruginosa* isolated in Auckland and many of these strains can transfer their antibiotic-resistance determinants for gentamicin and related antibiotics by conjugation. This supports the view that this mechanism is responsible for the emergence of resistant strains that are reducing the clinical value of gentamicin.

Whereas we have observed an increase in the proportion of isolates of *P. aeruginosa* resistant to gentamicin and our figures of 4% for 1974 and 8% for 1975 are similar to those of Holmes *et al.* (1974), who reported an increase in

the isolation of resistant strains from 4% to 8.9% in one year, Dean *et al.* (1978) found only 1.5% of their strains resistant to gentamicin.

In our study all strains that had a high level of gentamicin resistance transferred this resistance to recipient strains of *P. aeruginosa*. This differs from the experience of Holmes *et al.* (1974) who did not observe any transfer of resistance determinants from clinical isolates, and from Dean *et al.* (1978) who observed transfer of resistance determinants for streptomycin and tetracycline but not gentamicin, but agrees with the findings of Jacoby (1974a) and Bryan, Van den Elzen and Tseng (1972). Neither Jacoby (1974a) nor Bryan *et al.* (1972) commented on the degree of resistance of their donor strains nor did they find that such a high percentage of strains transferred resistance. Since then, Bryan *et al.* (1974) have reported that transfer of resistance was not demonstrable in strains for which the MIC of gentamicin was 6–100 µg/ml but that transferable R plasmids were present in strains for which the MIC was ≥ 100 µg/ml. This finding, that high-level resistance to gentamicin is plasmid-mediated, has been confirmed by Kontomichalou, Papachristou and Angelitou (1976) and by the present study. The mechanism of resistance in those strains with MICs of < 100 µg/ml is probably due to decreased permeability to gentamicin (Bryan, Haraphongse and Van den Elzen, 1976). The concomitant transfer of tobramycin resistance with gentamicin resistance was not an unexpected finding in light of the known mechanisms of resistance (Benveniste and Davis, 1973) and combined transfer has been observed by other workers (Kontomichalou *et al.*, 1976).

The isolation of a strain that was resistant to gentamicin, tobramycin and amikacin was unexpected. However, Kawabe, Naito and Mitsushashi (1975) have described a strain of *P. aeruginosa* resistant to amikacin and shown that the enzyme responsible was mediated by an R plasmid that also carried resistance to gentamicin, kanamycin and sulphanilamide. Our strain had a similar resistance pattern but the MIC of gentamicin was slightly higher—20 µg/ml compared with 3.1 µg/ml.

Like Bryan *et al.* (1974) and Jacoby (1974b) we found that no strain transferred resistance to *E. coli*. This suggests that the resistance determinants from gentamicin-resistant *P. aeruginosa* are unlikely to spread intergenerically within the hospital environment and the plasmids probably belong to incompatibility group P2 (Bryan *et al.*, 1973). Jacoby (personal communication) has confirmed that our plasmids belong to the Inc P2 group. The strains were isolated from several wards and apart from the contaminated bronchoscope suction tubing the only evidence of cross infection was in the haematology ward where four strains, half the total number isolated in this ward, were of the same pyocine type and serotype. We did not find evidence of cross infection to account for the increased incidence of gentamicin-resistant strains and this supports the conclusion that the spread of resistance in *P. aeruginosa* is plasmid mediated.

This survey revealed an increased incidence of gentamicin-resistant strains of *P. aeruginosa* in Auckland and demonstrated that plasmid-mediated aminoglycoside resistance can be transferred within the species. The findings

support the view that this mechanism is responsible for the emergence of resistant strains that are reducing the value of gentamicin in the management of infections caused by *P. aeruginosa*.

SUMMARY

Of 422 clinical strains of *Pseudomonas aeruginosa*, 23 (5.5%) were resistant to gentamicin; 19 were also resistant to tobramycin and sisomicin, and one was resistant to tobramycin, sisomicin and amikacin. Of the gentamicin-resistant strains, 20 were also resistant to kanamycin. Sixteen strains with a high level of resistance to gentamicin (MIC > 160 µg/ml) transferred all their resistance determinants to recipient strains of *P. aeruginosa* and four transferred some resistance determinants to *P. aeruginosa* but none transferred resistance to a recipient strain of *E. coli* K12. These results show that gentamicin resistance in strains of *P. aeruginosa* isolated in Auckland is mediated by R plasmids.

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