PREDOMINANCE OF *PSEUDOMONAS AERUGINOSA* SEROTYPE 11, PYOCIN TYPES 1 AND 10 IN SINGAPORE

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SYNOPSIS

***Pseudomonas aeruginosa* serotype 011 predominated in Singapore, accounting for 37.5% of the 112 clinical isolates tested. Serotypes 03, 04, 05 and 06 were the other common serotypes encountered (33.8% of the total). Pyocin types 1 (40.2%), 10 (22.3%) and 3 (13.4%) were the most prevalent. Sixteen percent of the total isolates were resistant to gentamicin and carbencillin while strains that were resistant to gentamicin, carbenicillin and tobramycin comprised 14.2% of the total. Ten clinical isolates (8.9%) were multiply-resistant to ticarcillin, netilmicin, gentamicin, carbenicillin and tobramycin. Most of the strains that showed multiple resistance were of serotype 011 (54% of strains showing multiple resistance), followed by serotype 07 (12.5%). These multiply resistant strains were isolated mainly from wound and urinary tract infections.**

INTRODUCTION

Pseudomonas aeruginosa is one of the most important pathogens involved in outbreaks of nosocomial infections (1, 2). Patients particularly susceptible to *P. aeruginosa* infections are those with immunocompromised host defense system such as those with cancer, acute leukemia and cystic fibrosis. Strains that had acquired multiple rersistance to antibiotics such as gentamicin, tobramycin and carbenicillin have made treatment with these antibiotics more difficult to manage. Epidemiological typing methods such as serogroup typing (3), phage typing (4) and pyocin typing (5) are available for tracing the sources and routes of transfer of strains involved in epidemic outbreaks. The present study was to determine the serotypes and pyocin types of clinical isolates of *P. aeruginosa* from hospitals in Singapore and to study their possible correlation with patterns of antibiotic resistance.

MATERIALS AND METHODS

Strains of *Pseudomonas aeruginosa*. One hundred and twelve strains of hospital isolates were received on Mueller-Hinton agar medium from the Pathology Laboratory of the Singapore General Hospital. These isolates were from various clinical specimens collected from seven local hospitals. *P. aeruginosa* strains were confirmed by growth on nalidixic acid-cetrimide agar (Oxoid, U.K.) and the determination of cultural and biochemical characteristics (6).

Antibiotic susceptibility test. Antibiotic susceptibility tests were performed in the clinical microbiology laboratory of the Pathology Department by the Bauer-Kirby single-disk diffusion method (7). The following disks were used: carbenicillin (100 μg), gentamicin (10 μg), tobramycin (10 μg), amikacin (30 μg), netilmicin (30 μg), ticarcillin (75 μg), sisomicin (10 μg) and polymyxin B (300 units).

Pyocin typing. All the 112 isolates of *P. aeruginosa* were typed by the cross-streaking method of Gillies and Govan (1966). The pyocin typing indicator strains 1-8, subtype set of A-E and four standard strains pp430, F1, Cree 7390 and Sands 3398 were kindly provided by Professor J.R.W. Govan, Department of Bacteriology, Medical School, University of Edinburgh.

Serological typing. Serotyping was carried out according to the International Antigenic Typing Scheme (IATS) with *Bacto-Pseudomonas aeruginosa* antisera from Difco Laboratories (8). The IATS antisera were directed against 17 serogroups which were type strains from all other commonly used *P. aeruginosa* serotyping systems. Types 1-12 were prepared using Hab's cultures 1-12, type 13 with Veron's 013 (Sandvik's type 11), type 14 with Verder and Evan's 05, type 15 with Lanyi's 012, type 16 with Meitert's type X strain and type 17 was ATCC 33364. Viable or unheated antigens were prepared by using cultures that were grown on Pseudomonas C-N Agar at 37°C for 18 to 24 h. When heated cells were to be used as antigens, cultures on Pseudomonas C-N Agar plates were washed and suspended in 10 ml of 0.85% NaCl solution. The bacterial suspension was autoclaved for 30 minutes at 121°C. The autoclaved antigens were filtered through cheese cloth. The bacterial suspension was centrifuged at 1,000 — 2,000 rpm for 10 minutes and the supernatant was discarded. The bacterial pellet was resuspended in 0.75 ml of merthiolate saline. The slide agglutination technique was applied for both viable and heated antigens.

RESULTS

In 1983, *P. aeruginosa* were isolated from 12.4% of clinical specimens submitted to the Pathology Laboratory from seven different hospitals in Singapore. Other isolates included *Klebsiella* species (18.8%), *Escherichia coli* (14.3%) and *Staphylococcus aureus* (13.1%). Most of the *Pseudomonas* strains were from surgical wounds (38.1%), 19.5% from burn wounds, 17.7% from urine and 12.4% from sputum and throat (Table 1).

Eighty-four isolates (75%) of the 112 strains were sensitive to all the antibiotics tested. Twenty-four strains (21%) were resistant to three or more antibiotics (Table 2). Strains that were resistant to gentamicin, carbenicillin and tobramycin comprised 14.2% of the total isolates. All strains tested were sensitive to polymyxin B.

Pyocin typing of the 112 *P. aeruginosa* strains with the eight indicator strains of Gillies and Govan showed four major and nine minor pyocin types. The results (Table 3) showed that a high proportion of strains (80%)

TABLE 1: SOURCE OF *P. AERUGINOSA* STRAINS

Source of Isolates	No. of Isolates	Frequency (% of Total)
Surgical wounds	43	38.3
Burn wounds	22	19.6
Urine	20	17.9
Sputum/Throat	14	12.5
Blood	4	3.6
Miscellaneous[a]	9	8.1

[a] Miscellaneous include clinical isolates from peritoneal fluid, eye, foot, ear, vagina, dressing, bed sore and catheter.

TABLE 2: FREQUENCY OF ANTIBIOTIC RESISTANCE IN CLINICAL ISOLATES OF *PSEUDOMONAS AERUGINOSA* IN SINGAPORE HOSPITALS

Pattern of Resistance[a]	No. of Isolates	Percentage (total)
Cb	3	2.7
Gm Cb	1	0.8
Gm Cb Si	2	1.8
Gm Cb Tb Si	4	3.6
Cb Tb Ne Ti	6	5.4
Gm Cb Tb Ne Ti	10	8.9
Gm Cb Tb Ne Ti An	2	1.8

[a] Resistance to carbenicillin (Cb), gentamicin (Gm), sisomycin (Si), tobramycin (Tb), netilmicin (Ne), ticarcillin (Ti) and amikacin (An).

TABLE 3: DISTRIBUTION OF PYOCIN TYPES IN 112 *P. AERUGINOSA* ISOLATES FROM SINGAPORE HOSPITALS

Pyocin Type[a]	Total (Percentage)
1	45 (40.2)
3	15 (13.4)
5	5 (4.5)
10	25 (22.3)
Others[b]	4 (3.6)
UC[c]	12 (10.7)
UT[d]	6 (5.4)

[a] Pyocin types according to the scheme of Gillies and Govan (1966).

[b] Pyocin types that are not represented in the Table.

[c] Unclassified types that showed variable inhibition patterns on repeated typing.

[d] Untypable types that showed inhibition patterns not included in Gillies and Govan's scheme.

could be assigned to relatively few pyocin types, namely, 1, 3, 5 and 10. Type 1 isolates were most common, constituting about 40% and occurring twice as frequently as type 10 (22%) isolates. Types 3 and 5 occurred at frequencies of 13.4% and 4.5% respectively. The minor pyocin types 6, 11, 22, 24, 43, 68 and 105 were rare, being encountered only once. Strains showing inhibition patterns that were not reported in Gillies and Govan's list of 105 pyocin types were classified as untypable (UT). Only 6 strains (5.4%) were untypable by this method. The occurrence of unclassified strains (UC) was significant (10.7%) and they differed in their inhibition patterns on each repeated typing. Typing was done at least 3 times by different persons to test the reproducibility of the method. Although Gillies and co-workers (5) compared the use of different media for typing and found no significant difference, we found that tryptone soya agar (TSA, Oxoid) supplemented with 5% defibrinated sheep blood gave a better background for reading of inhibition patterns. When typing was repeated by different persons, at least 35 strains could be assigned to 2 different pyocin types. It was found that weak pyocin producers tended to vary in their inhibition patterns on repeated typing. Especially significant was the variation between types 1 and 10 and types 3, 22 and 27. This was because these types differed by the reaction difference of only 1 indicator strain. Hence it would seem that scoring of results depended on assessing the amount of resistant growth of the test strains. We found even more striking differences when reproducibility of the results were compared using the subtype set of indicators. Only 68.8% of the isolates showed similar subtypes when evaluated by different persons on separate occasions.

As most of the strains belonged to only a few major pyocin types, further typing with the five sub-type indicator strains showed that pyocin type 1 (Table 4) could be further differentiated into 7 subtypes. Subtype h was most predominant, accounting for 44% of the type 1 strains.

The distribution of O serotypes in Singapore hospitals is shown in Table 5. The predominant serotype was 011 which accounted for 37.5% of all isolates. Serotypes 03, 04, 05 and 06 were the other common serotypes encountered (33.8% of the total). The number of non-typable isolates accounted for only 8.9% of all isolates. No isolate of serotypes 013, 014, 015 and 017 was encountered. Serotypes 01, 09 and 016 were each represented only once. Only two isolates (1.8%) showed poly-agglutination. These strains were also classified as non-typable strains. No isolate was found to be self-agglutinating. When an isolate showed agglutination in two antisera, the test was repeated using heated antigens. There was good correlation (92%) between the use of heated and viable antigens. When different results were observed with viable and heated antigens, the isolates were serotyped according to the reactions obtained with the heated

TABLE 4: DISTRIBUTION OF THE MAJOR PYOCIN TYPES INTO SUBTYPES

	Subtype	No. of Isolates (Percentage)
Pyocin 1	a	1 (2.2)
	b	7 (15.6)
	c	6 (13.3)
	d	5 (11.1)
	h	20 (44.4)
	g	1 (2.2)
	x	1 (2.2)
	UC[a]	3 (6.7)
	UT[b]	1 (2.2)
Pyocin 3	a	1 (6.7)
	e	7 (46.7)
	1	5 (33.3)
	n	1 (6.7)
	UC[a]	1 (6.7)
Pyocin 5	f	3 (60)
	k	1 (20)
	UC	1 (20)
Pyocin 10	a	4 (16.0)
	b	2 (8.0)
	d	1 (4.0)
	h	7 (28.0)
	UC[a]	8 (32.0)
	UT[b]	3 (12.0)

[a] Unclassified subtypes due to variable inhibition patterns on repeated typing.

[b] Untypable subtypes showed inhibition patterns not reported by Gillies and Govan (1966).

TABLE 5: DISTRIBUTION OF SEROTYPES OF *P. AERUGINOSA* IN SINGAPORE

Serotype	No. of Isolates	Frequency (% of Total)
1	1	0.9
2	2	1.8
3	9	8.0
4	12	10.7
5	9	8.0
6	8	7.1
7	2	1.8
8	2	1.8
9	1	0.9
10	6	5.4
11	42	37.5
12	5	4.5
13	0	0
14	0	0
15	0	0
16	1	0.9
17	0	0
NT[a]	10	8.9
PA[b]	2	1.8

[a] NT denotes non-typable strains

[b] PA denotes polyagglutinable strains.

TABLE 6: DISTRIBUTION OF SEROTYPES IN RELATION TO ANTIBIOTIC RESISTANCE AND SITES OF INFECTION

Serotype	No. of Isolates	Resistance Pattern[a]	Site of Infection[b]
4	2	Cb^r (1)	Urinary tract (1)
		$Gm^rCb^rTb^rNe^rTi^r$ (1)	Urinary tract (1)
6	1	Cb^rGm^r	Urinary tract (1)
7	3	$Gm^rSi^rCb^r$ (1)	Urinary tract (1)
		$Gm^rSi^rCb^rTb^r$ (1)	Urinary tract (1)
		$Gm^rCb^rTb^rNe^rTi^r$ (1)	Wound 1
8	1	$Gm^rSi^rCb^rTb^r$	Respiratory tract (1)
10	1	$Gm^rCb^rTb^rNe^rTi^r$	Wound 1
11	16	Cb^r (3)	Urinary tract (1)
			Sputum (1) Wound (1)
		$Gm^rSi^rCb^r$	Urinary tract (1)
		$Cb^rTb^rNe^rTi^r$ (6)	Wound (6)
		$Cm^rCb^rTb^rNe^rTi^r$ (4)	Wound (3) Urinary tract (1)
		$Gm^rCb^rTb^rNe^rTi^rAn^r$ (2)	Wound (2)
12	3	$Gm^rCb^rTb^rNe^rTi^r$	Wound (2) Urinary tract (1)
NT	2	$Gm^rSi^rCb^rTb^r$	Urinary tract (2)

Numbers in parentheses are the numbers of isolates that display the particular antibiotic resistance pattern[a] and the number of isolates associated with the sites of infection.[b]

antigens. The reproducibility of serotyping results was checked by assigning different persons to perform the agglutination test. Good reproducibility (92.9%) of the results was obtained.

Strains belonging to serotype 04, 06, 07, 08, 010, 011, 012 and two strains of non-typable serotype were found to be resistant to several of the commonly used antibiotics against *Pseudomonas* infections (Table 6). These resistant strains were isolated mainly from wound and urinary tract infections. Sero-type 011 accounted for 56% of the total number of strains that were antibiotic-resistant. Of the 25 strains that showed multiple antibiotic resistance, 13 (52%) were serotype 011. Six serotype 011 strains with the $Cb^rTb^rNe^rTi^r$ resistance pattern, three 011 strains with the $Gm^rCb^rTb^rNe^rTi^r$ resistance pattern and one 011 strain with the $Gm^rCb^rTb^rNe^rTi^rAn^r$ resistance pattern were all isolated from burn wound infections from the same burns wards of a single hospital.

DISCUSSION

P. aeruginosa has been found to be an opportunistic pathogen associating with about 10%-12.5% of all nosocomial infections (1,2,9). In a survey of 417 nosocomial infections associated with *P. aeruginosa*, Sherertz and Sarubbi (10) found this organism to be the second most common pathogen isolated from a University teaching hospital. In our study, *P. aeruginosa* was the fourth most common pathogen isolated from hospital specimens. Since we did not trace the source of the infections, no conclusions could be drawn as to whether these infections were common source outbreaks or cross-infection outbreaks and whether the strains involved were endemic or epidemic strains. However, the isolation of 18 strains of serotype 011 from the burns ward of a single hospital out of a total of 67 isolates submitted by the same hospital does suggest that these strains were involved in either common source outbreaks or cross-infection outbreaks.

Although we were able to type 94.6% of our isolates by the cross-streaking method of Gillies and Govan (5), the reproducibility of this method was variable because of the subjective nature of interpretation of the inhibition patterns. Another disadvantage of the pyocin typing method is the time required to screen through the tables of inhibition patterns (11), to assign an unknown clinical isolate to a particular pyocin type and subtype. Pyocin typing is also a less rapid method than serotyping. We found serotyping a more convenient and reproducible method. Pooled antisera (12) were not used although these could reduce the time required for serotyping an unknown organism.

Distribution of serotypes has been found to differ with respect to geographical locations. Serotype 02 was the most common serotype in extra-intestinal pathological specimens throughout the world, with 06 being the next common (3). Both serotypes were isolated at rather low frequencies in Singapore, 1.8% and 7.1% of the total number of strains respectively. We found the predominance of 011 (37.5%) which had also been shown to occur more frequently in India (23-24.1%), Japan (16.3%) and the United States (8.9-17.7%) than in Europe (3.7-7.1%). Recent serotyping of *P. aeruginosa* associated with nosocomial infections in the U.S.A. by Sherertz and co-workers (10) showed that serotype 06 was the most common serotype (20.7%), followed by 01 (16.8%) and 011 (16.8%). Serotype 011 was found to cause 9 of 17 (53%) single strain outbreaks although it formed only 8% of the endemic isolates (13). This serotype was involved in common source outbreaks where contaminated urological instruments, disinfectants and contaminated whirlpools in motels (13,14,15) were shown to be the sources of the organism. *P. aeruginosa* serotype 011 has been reported to cause skin rashes (16) and was also associated with otitis externa in deep-sea divers (17). This report further supports the findings of Farmer and co-workers (13) that serogroup 011 is significant in causing hospital infectioins.

Sherertz and co-workers (10) found a statistically significant association between certain serotypes and antibiotic resistance patterns. There was correlation between serotype 06 to carbenicillin, gentamicin and tobramycin resistance and serotype 011 could be correlated to carbenicillin resistance. We observed a correlation between serotype 011 with resistance to carbenicillin and tobramycin or carbenicillin, gentamicin and tobramycin. These resistance patterns differed from those reported by Legakis and co-workers (12) in clinical isolates of *P. aeruginosa* isolated from Greece. Most of their strains of serotypes 06 and 011 were sensitive to gentamicin and carbenicillin. Instead, serotype 012 occurred most frequently amongst strains that were resistant to these two antibiotics.

Antibiotic therapy and *Pseudomonas* vaccines administered either alone, or in combination with hyperimmune globulins, have been used for treatment or prophylatic prevention of *Pseudomonas* infections. (9) For antibiotic therapy, there is a need to continually monitor the antibiotic susceptibility patterns of clinical isolates. However, *in vitro* sensitivity of clinical isolates to a particular antibiotic does not necessarily correlate with *in vivo* therapeutic efficacy. This was the case for polymyxin B. Most isolates of *P. aeruginosa* were found to be inhibited *in vitro* by very low concentrations of polymyxin B (median, 0.78 μg/ml) (18) which was also observed with our local isolates. Amikacin had been found to be effective against *P. aeruginosa* isolates that were resistant to other aminoglycosides. (9) The frequency of isolation of amikacin resistant strains in our present study was low (1.8%) when compared with those resistant to gentamicin (16.8%). Ticarcillin is a relatively new anti-pseudomonal penicillin but we have isolated strains resistant to this antibiotic. Careful monitoring of its usage is needed to prevent widespread resistance to this antibiotic.

Resistance to aminoglycosides can arise from either enzymatic modification of the antibiotics or decreased membrane permeability to them. If the genes encoding enzymes for acetylation, adenylation or phosphorylation are carried on plasmids, dissemination of plasmids will cause widespread resistance. This is also one of the disadvantages of using antibiograms as an epidemiological tool. It is not known whether the predominance of serotype 011 could be due to the presence of plasmids which encode for multiple resistance (19) or altered outer cell wall and membrane structures (9).

Serological typing of clinical isolates of *P. aeruginosa* is more relevant to the use of vaccines than pyocin typing. Two vaccines that have been developed were the polyvalent vaccine (PEV-01) (20) and a heptavalent vaccine (21). The polyvalent vaccine was made up of 16 serotypes whereas the heptavalent vaccine contained 7 serotypes. These 7 serotypes were found in more than 90% of the *Pseudomonas* infections in many insstitutions (21). However, the use of the heptavalent vaccine may not be relevant in geographical locations where these seven serotypes are not encountered. Thus, there is a need for serological typing of local *P. aeruginosa* isolates.

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