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Epidemiology of *Pseudomonas aeruginosa* in a Burns Hospital: Evaluation of Serological, Bacteriophage, and Pyocin Typing Methods[1]

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In a retrospective study 36 cultures of *Pseudomonas aeruginosa*, isolated from patients with fatal *Pseudomonas* burn wound sepsis and from burned patients with nonfatal *P. aeruginosa* infections, were used to evaluate the consistency and reliability of serological, phage, and pyocin typing as epidemiological tools. Frequency distributions of positive reactions were analyzed by a computer in a 3-way chi-square test, and a high degree of consistency was demonstrated for each method. From these data, 75% of the cultures were differentiated by serological, 90% by phage, and 100% by pyocin typing. There was no significant difference among organisms isolated from fatal cases of burn wound sepsis and organisms from patients with nonfatal infections ($\chi^2 = 0.3418$; $P = 0.9870$). The combined typing system was a sensitive and reliable epidemiological tool for intraspecific differentiation of *P. aeruginosa*.

Many types of bacteria have been associated with burn wound sepsis, but infections resulting from gram-negative bacteria are often severe and difficult to control. Among the gram-negative bacteria, *Pseudomonas aeruginosa* is the predominant cause of fatal burn wound sepsis (10, 11, 16, 17).

Carriers among patients and personnel, infected patients, and a variety of inanimate objects may serve as reservoirs or vehicles for transmission of *P. aeruginosa* in the hospital environment. To identify such sources and routes of transmission for this infectious agent, definitive methods must be used for its differentiation at the intraspecific level. Several methods for serological, bacteriophage, and pyocin typing of *P. aeruginosa* are available, but their usefulness as epidemiological tools remains questionable. In this study, a retrospective epidemiological investigation was conducted to evaluate serological, bacteriophage, and pyocin typing methods for consistency and reliability.

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MATERIALS AND METHODS

Test organisms. On the basis of clinical histories of patients, 36 preserved cultures of *P. aeruginosa*, isolated from clinical materials in the Microbiology Laboratory, Shriners Burns Institute, were divided into two groups: (i) organisms isolated from patients with documented fatal *Pseudomonas* burn wound sepsis (Table 1) and (ii) organisms isolated from patients with nonfatal *P. aeruginosa* infections (Table 2). Subsequently, these cultures were used in experiments designed to determine the reliability of serological, phage, and pyocin typing systems.

Chemicals and media. Unless specified differently, all chemicals were of reagent or pure grade, and media were of a quality recommended for use in microbiological and clinical laboratories. Mitomycin-C (crystalline) was obtained from Sigma Chemical Company, St. Louis, Mo.

Serological typing. Rabbit antiserum to each of the seven Fisher immunotypes (6) were kindly supplied by H. B. Devlin, Parke, Davis & Co., Detroit, Mich.

Each culture was grown on a slant of Mueller-Hinton agar (Difco) overnight in a water bath at 30 C. Growth was removed from each slant with a sterile cotton-tipped applicator and suspended in saline to form a homogenous suspension. Then, one drop of antiserum was mixed with each bacterial suspension on a microscope slide and observed for agglutination.

TABLE 1. *Cultures of Pseudomonas aeruginosa isolated from patients with fatal burn wound sepsis*

Patients				Cultures		
Code	Date admitted	Postburn days on admission	Deceased date	No.	Isolation date	Source
J.V.	2/23/70	1	5/6/70	10,483	5/5/70	Blood
C.N.	2/17/70	13	3/4/70	9,221	3/4/70	Blood
M.D.	1/23/69	17	2/23/69	3,550	2/15/69	Blood
				3,770	2/23/69	Right upper thigh
M.L.	12/31/68	6	1/12/69	3,055	1/12/69	Catheter
				3,037	1/12/69	Blood
R.K.	6/25/68	6	7/14/68	1,453	7/15/68	Burn tissue
K.W.	4/19/68	8	5/16/68	794	5/16/68	Blood
W.T.	12/16/68	3	1/8/69	2,999	1/8/69	Blood
K.T.	9/20/69	15	10/31/69	6,760	10/30/69	Deep muscle, Right leg
C.H.	6/26/68	13	7/19/68	1,497	7/19/68	Blood
				1,533	7/20/68	Peritoneal Cavity
C.D.	8/14/68	7	8/23/68	1,764	8/23/68	Blood
				1,775	8/23/68	Pericardia
S.W.	4/28/70	12	5/21/70	10,796	5/21/70	Heart
C.O.	5/13/70	2	7/19/70	11,126	6/22/70	Blood

TABLE 2. *Cultures of Pseudomonas aeruginosa isolated from patients with nonfatal burn wound infections*

Patients				Cultures		
Code	Date admitted	Postburn days on admission	Discharged date	No.	Isolation date	Source
S.N.	7/23/68	16	2/21/69	1,641	8/8/68	Blood
R.T.	3/8/69	16	5/29/69	4,866	4/25/69	Foley catheter
				4,223	3/22/69	Blood
L.W.	1/5/68	0	3/23/68	3	2/19/68	Wound
G.W.	4/1/68	1	6/13/68	624	4/26/68	Left arm
J.W.	6/4/68	10	7/12/68	1,216	6/20/68	Left arm
C.W.	6/20/69	9	8/20/69	5,744	7/13/69	Heterograft Right chest
M.V.	10/23/68	2	2/9/69	2,271	10/23/68	Left face
				2,492	11/18/68	Urine
B.H.	9/7/68	7	11/30/68	1,897	9/9/68	Stool
				1,959	9/15/68	Chest
T.B.	1/3/69	2	4/4/69	3,580	2/16/69	Left thigh
J.B.	5/7/69	5	7/21/69	5,434	6/13/69	Urine
J.C.	9/13/68	27	10/31/68	2,189	10/14/68	Right chest
R.C.	7/13/69	0	8/5/69	5,835	7/22/69	Left thigh
S.H.	7/11/69	14	9/3/69	5,827	7/20/69	Left leg
C.H.	3/21/68	36	4/24/68	4,883	4/25/69	Left arm
L.H.	8/11/69	246	10/19/69	6,033	8/18/69	Left arm
R.M.	11/6/68	59	12/17/68	2,409	11/6/68	Urine
D.A.	5/20/70	13	7/3/70	11,127	6/22/70	Urine

Bacteriophage typing. Twenty-two phages and their homologous propagating strains were kindly supplied by M. T. Parker (Cross Infection Reference Laboratory, Central Public Health Laboratory, Colindale, N.W. 9, London, England). Test cultures were typed by five previously described methods (8, 7, 13–15) to select a set of uniform conditions for such variables as medium composition, culture age, and incubation temperature. Subsequently, the following modified procedure was used in this study.

Lyophilized phages were reconstituted with 0.5 ml of Trypticase soy broth (TSB) and propagated in their homologous host strains in TSB, TSB plus $CaCl_2$ (400 μg/ml), sodium lactate medium (SLM), and on Trypticase soy agar (TSA) plus 0.7% purified agar. Plates were incubated for 12 to 14 hr at 32 C,

frozen for 3 hr, and allowed to thaw at room temperature. Then plates were tilted, and the supernatant fluid was transferred to sterile tubes. Propagating strains were killed with chloroform (0.3 ml/5 ml of culture) or by heat in a water bath at 58 C for 2 hr. Host bacterial cells were sedimented by centrifugation at 3,000 rev/min for 30 min, and phages were harvested from the supernatant fluid. Subsequently, the routine test dilution (RTD) for each phage strain was determined on its respective host.

Each test culture was grown in TSB for 2 hr at 32 C and inoculated onto the surface of a TSA plate (containing 1% purefied agar) for confluent growth. Open plates were dried at room temperature for 1 hr. Then one drop of each phage RTD was added to each test culture. After phages had absorbed to the medium, plates were incubated at 32 C for 12 to 14 hr. Then lytic patterns were scored as follows: confluent lysis, +++; 50 to 100 plaques, ++; 20 to 50 plaques, +; fewer than 20 plaques, ±. Only +++ and ++ scores were recorded as type reactions.

Pyocin typing. Twenty-seven pyocin indicator strains and 24 pyocin producer strains were kindly supplied by J. J. Farmer III, National Institutes of Health, Bethesda, Md.

In preliminary studies, test cultures were typed by both pyocin production and pyocin sensitivity as previously described (5). Subsequently, we modified the qualitative method of Zabransky and Day (20) and typed our test cultures by pyocin production against the pyocin indicator strains of Farmer and Herman (5). Test cultures were grown in TSB at 32 C for 2 to 4 hr. Then TSA plates containing 0.1% sodium citrate, 0.1% dipotassium hydrogen phosphate, and 10^{-5} M iodoacetic acid were inoculated with each TSB culture in a central streak across the surface of the medium. The three chemicals were added to suppress the action of pyocin-inactivating substances (19). Plates were incubated for 12 to 14 hr at 32 C, after which they were inverted, and 0.3 ml of chloroform was added to the lid of each plate to kill test cultures. Cultures were exposed to chloroform vapors for 30 min. Then residual growth was removed from the agar surface with a sterile swab. Plates were cross-streaked with indicator strains and incubated at 32 C for 12 to 14 hr, after which zones of growth inhibition in indicator strains were recorded as positive (+) or negative (−). These 27 reactions were then converted into a 9-digit number according to a mnemonic for type designation (4).

Evaluation of typing methods. All *P. aeruginosa* cultures (Table 1 and 2) were given random numbers and kept in assigned order in subsequent experiments. Cultures were typed four times at intervals of 6 to 8 weeks with each of the three methods. Then, the frequency of positive reactions was compared among methods and between groups of cultures.

RESULTS

Serological typing. No changes were made in procedures for serological typing of *P. aeruginosa*. Test cultures were typed by the slide agglutination method with Fisher's seven antisera (6). The frequency of positive reactions in representative cultures is shown in Table 3.

Bacteriophage typing. On the basis of preliminary investigations, four media influenced the development of plaques in their respective homologous strains. Highest and most uniform titers were obtained for 21 phages when propagated on TSA by the soft-agar overlay method. Phage 31 did not produce high-titer RTD on any media. High titers were produced on TSA without added $CaCl_2$, which is a requirement for many phages. The lytic patterns for these phages have remained stable for over a year. Differences in lytic patterns were observed when test cultures were typed on these media. Most consistent reactions were obtained when cultures were typed on TSA (Table 4).

Pyocin typing. In preliminary studies, comparable results were obtained from pyocin typing of test cultures by the qualitative method of Zabransky and Day (20) and by the quantitative method of Farmer and Herman (5). However, titers of liberated pyocins lost their activity when stored at 2 to 4 C. As a result of this observation, a modified qualitative procedure was used to detect pyocin production from test cultures against 27 pyocin indicator strains. The frequency of positive reactions in indicator strains is shown in Table 5.

Evaluation of typing methods. A computer was used to analyze the data in a three-way chi-square test to determine whether cultures differed with respect to frequency distribution of positive reactions between groups (Tables 1 and 2) and among typing methods. There was

TABLE 3. *Consistency of serological reactions of cultures*[a]

No.	Positive reactions[b]							
	0	1	2	3	4	5	6	7
11,126		4						
1,775								4
10,796			4					
4,223		4						
6,033		4						
1,497				4				
10,483	4							
2,189		4						
9,221			4					
3,037	1		3					
2,999	1						3	
1,216		4						
1,897	1	1				2		

[a] Cultures were typed by the slide agglutination method with Fisher's seven antisera (reference 6).

[b] Four tests were done for each culture.

TABLE 4. *Consistency of phage reactions*

Culture No.	Frequency of positive reactions[a]																					
	0	7	16	21	24	31	44	68	73	F7	F8	F10	109	119X	352	1214	M4	M6	C21	C11	C18	(188/1)	2
11,126		4		4				3	1					4							1	1	4
1,775										2				1								2	
10,796					1																	4	
4,223											1											2	
6,033	4																						
1,497													1			4						3	
10,483										1													
2,189		4							3				2				4			4		4	4
9,221				1																		1	
3,037				4				2						1				1			3	3	
2,999		4		3				3					2	1							1	4	1
1,216		4							4				2	4	3	3		1		4			4
1,897																		1				4	

[a] Four tests were done for each culture.

TABLE 5. *Consistency of pyocin reactions*

Culture No.	Frequency of positive reactions[a]																									
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
11,126			1	2	2	3	1	1										1							1	1		1
1,775			3		3	3	4	3		1	1		2	1	3	2		3	1	3	1	2	3	3	2	2		2
10,796			3		3	4	4			1		1	1	4	4			2	2	2	1		4		1	4		2
4,223			2	1	2	3	4			1					2	1	2	2	2	2			2	2	2	3		2
6,033				2	4			3	1	2				3		2	3							2	3			
1,497			1		2	2	2	1		4	1	1			1	1		2						2	3	2		3
10,483			3		4	4	4			3			1	4	4		2	3	3	3			4	3	3	3		3
2,189				1	4	2		4	2	4				4		4	3			2			2	4	4			
9,221			3		4	4	3			4				1	3	2	4	4	4	4	1		4	4	4	4		4
3,037			2		3	2	2			3					4		2	3	3	1			2	2	2	2		2
2,999		4	1		3	3	2	2		4					2	2	2	1	4	2			1	3	4	1		1
1,216					4			3		4				4		2	2			1				3	1	1		
1,897			3		3	4	4	3	2	3	1	1		1	3	2		3		2		1	3	1	4	3		2

[a] Four tests were done for each culture.

no significant difference between groups of organisms with respect to the distribution of positive reactions for a separate typing method or for all three typing methods ($\chi^2 = 0.3418$; $P = 0.9870$). However, there was a highly significant difference among typing methods for both groups of organisms and for each group separately ($\chi^2 = 244.9396$; $P = 0.0000$).

Type reactions for all three methods were made on the basis of the following criteria: 2 to 4 positive reactions per culture in four experiments were designated as major reactions; and 0 to 1 positive reactions per culture were designated as nontypable. With these criteria, 90% of test organisms were differentiated by serological typing, 75% by phage typing, and 100% by pyocin typing (Tables 6 and 7).

As a combined system, nine cultures were typable by only serology or phage, and two cultures (10483 and 1533) were nontypable by both methods. However, these nontypable cultures were identified by pyocin production. They were from different patients (Table 1), and each was identified by different mnemonics for strain designations. In the combined system, 100% of our cultures were differentiated at the intraspecific level.

DISCUSSION

The continuing increase in *P. aeruginosa* infections in hospitals is similar, in many ways, to the problem of *Staphylococcus* infections of approximately 15 years ago. However, the development of a standardized procedure for phage typing of staphylococci (2) proved to be an invaluable aid in understanding the epidemiology of *Staphylococcus* infections. Unfortunately, similar successes with typing methods for *P. aeruginosa* cannot be acclaimed. Several procedures have been described for typing *P. aeruginosa* by serology (6,

7, 9, 18), phages (8, 13–15), and pyocins (1, 3, 5, 12, 20), but their value as epidemiological tools remains questionable. In this report we have suggested a combined typing system for intraspecific identification of *P. aeruginosa*.

Each of the typing procedures was evaluated for consistency as a single method and as a component of a combined typing system. Chi-square analysis revealed significant differences among serological, phage, and pyocin typing methods (χ^2 = 244.9396; P = 0.0000). The large proportion of minor reactions in the method for pyocin typing contributed to the significant difference that was noted among methods. The proportion of reactions per culture (0 to 1, 2, 3 and 4) were lowest in serological types and highest in phage types.

TABLE 6. *Intraspecific identification of P. aeruginosa cultures isolated from patients with fatal burn wound sepsis*

Culture No.	Serological type	Pyocine type (mnemonic)	Bacteriophage type (lytic pattern)
10,483	NR	617 821 515	NR[b]
9,221	2	617 841 513	NR
3,550	5	813 846 513	1214/(188/1)
3,770	5	611 816 513	1214/(188/1)
3,055	2	618 861 513	21/68/C11/(188/1)
3,037	2	617 861 813	21/68/C11/(188/1)
1,453	4	858 888 888	7/21/44/68/1214/(188/1)
794	1/2[a]	887 586 847	7/68/109/352/C18/(188/1)/2
2,999	6	513 843 548	7/21/68/109/(188/1)
6,760	2	617 861 213	NR
1,497	3	817 886 843	1214/(188/1)
1,533	NR	873 884 887	NR
1,764	7	613 848 573	NR
1,775	7	615 746 327	F7/(188/1)
10,796	2	617 824 553	(188/1)
11,126	1	728 888 888	7/21/68/119X/(188/1)/2

[a] Cross-reactions.
[b] NR = no reactions.

Test cultures were selected from different sources to determine whether correlations could be made between types and source. Data from chi-square analysis revealed no significant difference among organisms isolated from fatal cases of burn wound sepsis and organisms from nonfatal infections (χ^2 = 0.3418; P = 0.9870).

Due to the monospecificity of serological types, strain differentiation was stable and highly consistent. Phage and pyocin types are seldom monospecific, and it is difficult to interpret the significance of specific variations in pattern reactions. A gain or loss of a single reaction will result in a different lytic pattern. Several pairs of cultures (3550 and 3770, 3055 and 3037, 4866 and 4223) from similar sources showed identical reactions in phage and serological types. In contrast, other pairs (1497 and 1533, 2271 and 2497, 1897 and 1959) from similar sources showed different reactions in both phages and serological types. On the basis of

TABLE 7. *Intraspecific identification of P. aeruginosa cultures isolated from patients with nonfatal burn wound sepsis*

Culture No.	Serological type	Pyocine type (mnemonic)	Bacteriophage type (lytic pattern)
1,641	3	888 888 848	21/44/68/F8/119X/1214/(188/1)
4,866	1	617 864 513	(188/1)
4,223	1	618 811 513	(188/1)
3	3/7[a]	114 886 321	NR[b]
624	NR	887 888 888	7/44/F8/109/119X/352/1214/C11/2
1,216	1	853 865 568	7/73/109/119X/352/1214/C11/2
5,744	1	853 865 568	7/109/M4/(188/1)/2
2,271	NR	648 825 858	7/44/68/F8/109/1214/(188/1)/2
2,492	1	648 868 857	7/16/44/68/F8/109/1214/C18/C11/(188/1)/2
1,897	5	611 846 533	(188/1)
1,959	1	613 836 513	7/(188/1)
3,580	1	751 166 144	7/73/109/119X/M4/C18/(188/1)/2
5,434	3	833 835 543	1214/(188/1)
2,189	1	821 836 518	7/73/109/M4/C18/(188/1)/2
5,835	1	851 515 868	7/21/68/(188/1)/2
5,827	5	613 565 313	(188/1)
4,883	3	817 868 847	NR
6,033	1	753 835 848	NR
2,409	2	717 861 547	(188/1)
11,127	2	617 761 513	NR

[a] Cross-reactions.
[b] NR = no reactions.

similarities in all reactions for these pairs of cultures in the three typing systems, strain identification can be made with greater confidence. Therefore, we used the three methods simultaneously to differentiate the cultures at the intraspecific level. The percentage typable in each method was: serological 90%, phage 75%, and pyocin 100%.

On the basis of these data, we suggest typing cultures simultaneously with serology and phage and by pyocin as an adjunct to the other methods for identification of nontypable strains. As separate methods, serological and phage typing had the advantage of being simple to perform, but they were limited as epidemiological tools by the fact that 10 to 25% of cultures were nontypable. Pyocin typing had merits when used as an adjunctive method for resolution of nontypable cultures because 100% of the cultures produced pyocins. However, pyocin typing is limited by the time required to complete the test and by variations in lytic patterns, as observed in cultures from similar sources. Because of the lack of stability in isolated pyocins by mitomycin-C induction, the qualitative typing test was preferred. This combined system (which can be completed in four days) enabled us to make strain designations with greater confidence. The application of this system in a prospective study of *P. aeruginosa* infections is being reported in the article that follows.

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