JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1986, p. 1017–1022
0095-1137/86/121017-06$02.00/0

Vol. 24, No. 6

Improved, Computer-Generated System for Pyocin Typing of *Pseudomonas aeruginosa*

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Received 23 June 1986/Accepted 26 August 1986

We applied numerical clustering algorithms to the selection of a new indicator strain set for the pyocin typing of *Pseudomonas aeruginosa*. The new indicator set is composed of selected indicator strains from the sets described in 1966 by Gillies and Govan (J. Pathol. Bacteriol. 91:339–345) and in 1974 by Jones, Zakanycz, Thomas, and Farmer (Appl. Microbiol. 27:400–406) and is designated the G-F set. This indicator set consists of 14 indicator strains which typed 99.5% of 114 test cultures, has a high degree of discrimination (10 patterns encompass 50% of the test strains), and provides 62.3% reproducibility of the same typing pattern in duplicate tests done on different days. The G-F set of indicator strains provides slightly higher percentages of typable cultures than either of the other two sets, has greater discriminatory capability, and is more reproducible than they are. We recommend that the G-F set of indicator strains be used instead of the two other sets for pyocin typing of *P. aeruginosa*. We also tested a recently described overlay procedure for pyocin testing of *P. aeruginosa* and found it to be superior to previous methods in that it is easier to perform, it provides answers in only 24 h instead of 48 h, and it can be used to type mucoid strains (which previous techniques could not readily do). Thus, the application of numerical clustering algorithms and use of a revised typing procedure have produced an improved system for pyocin typing of *P. aerguinosa*. Similar procedures may be applicable to other typing systems.

Epidemiologic typing of strains of *Pseudomonas aeruginosa* can be done by a variety of techniques, including biotyping (7, 27), serologic typing (9, 27, 29), bacteriophage typing (5, 6, 14, 16), pyocin typing (3, 8, 14, 16, 20, 25, 30), and antimicrobial susceptibility patterns (7, 8).

Of these procedures, antimicrobial susceptibility is most commonly performed, but serotyping is most often used for epidemiologic purposes. Pyocin typing may be used in conjunction with serotyping because it generally yields a higher percentage of typable strains and is more discriminating (8, 14). Thus, pyocin typing may differentiate among serologically identical strains. Unfortunately, pyocin typing is less reproducible than serotyping, and this, along with the inclination of most investigators to rely more on serologic patterns than on pyocin patterns, makes pyocin typing less available.

The two major techniques used for typing by pyocin production are those of Gillies and Govan (18, 21) and Jones et al. (25). In their original form, these two systems shared a common problem in that at least 48 h was needed to produce results. However, Fyfe, Harris, and Govan (17) recently described a modified procedure which requires less time and can be applied to mucoid strains as well.

If pyocin typing could be made more reproducible, it might be of more value than serologic typing. However, the selection of a set of indicator strains for pyocin typing is always difficult and often made quite subjectively, primarily because there are a variety of factors which must be considered, such as stability of cultures, lack of bacteriophages, ease of reading zones of inhibition, etc. Further, the existence of two major, but different, methods and sets of indicator strains has not contributed to standardization of techniques for pyocin typing of *P. aeruginosa*.

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We compared the two most commonly used sets of indicator strains and attempted to develop a new set which might prove to be more reproducible and discriminating than either set alone. Our approach to choosing a new set of indicator strains was to apply numerical clustering algorithms (1, 23, 24). This statistical technique forms clusters based on a measure of association or similarity among the indicator strains. If it were found to be useful in this application, then we considered that it might be applied to the selection of other sets of cultures, bacteriophages, etc. We also tried and confirmed the value of the modified pyocin typing procedure described by Fyfe et al. (17).

MATERIALS AND METHODS

Cultures. Indicator strains were obtained from J. R. W. Govan, University of Edinburgh, Edinburgh, Scotland, and J. J. Farmer III, Centers for Disease Control (CDC), Atlanta, Ga. The thirteen Govan strains, labeled 1 to 8 and A to E, were used because they were used in a recent modification of the original technique for pyocin typing (17). These cultures were lyophilized and stored at 4°C. The 18 Farmer strains, labeled ALA 1 to 18, were used because they had been chosen originally by use of a computer program (25) and because we hoped to use them in conjunction with or instead of certain Govan strains to obtain a better set of indicator strains. These cultures were frozen in defibrinated sheep blood at −70°C. Working cultures were maintained on blood agar base (BBL Microbiology Systems, Cockeysville, Md.) slants at room temperature. Subcultures from the lyophilized and frozen stock cultures were made monthly to fresh agar slants.

We obtained 416 clinical *P. aeruginosa* strains from culture collections at the CDC. Of these cultures, 390 were from human and 26 were from environmental sources. A majority of the cultures were from one hospital, but many serologic

and pyocin types were represented. The identity of all of these isolates had been confirmed previously in CDC laboratories by conventional methods (12). None of the strains in this collection was mucoid. These cultures were maintained in a Trypticase-soy (BBL) agar-based semisolid medium at room temperature.

Ten mucoid strains of *P. aeruginosa* were obtained from Robert E. Weaver (CDC) to see whether they could be typed by the revised method.

Pyocin typing. The method used for typing *P. aeruginosa* by pyocin production was that of Fyfe et al. (17). The strains to be typed were streaked for isolation onto nutrient agar (Oxoid Ltd., London, England) and incubated at 37°C overnight. Isolated colonies were then used to prepare a bacterial suspension in 1.0 ml of sterile physiological saline equivalent to the density of a 0.5 McFarland standard (about 10^8 organisms per ml). A Steers replicator with 24 stainless steel pins 3 mm in diameter and spaced 11 mm apart was used to inoculate the bacterial suspensions onto a set of tryptone soya agar (Oxoid) plates, each containing 10 ml of medium. After the spots dried, the plates were incubated at 30°C for 6 h. A 7-cm-diameter circular filter paper (Whatman no. 1; Whatman, Inc., Clifton, N.J.) was placed in the center of a glass petri dish lid and saturated with approximately 1 ml of chloroform. The agar plate was then inverted over the filter paper for 15 min to allow the bacteria to be killed by the chloroform vapor (2). The plates were then exposed to air for 15 min to ensure complete removal of any residual chloroform vapor.

Meanwhile, indicator strains were grown in nutrient broth (Oxoid no. 2) for 4 h at 37°C without agitation. To 2.5 ml of melted semisolid agar (containing 1% peptone [Difco Laboratories, Detroit, Mich.] in 0.5% agar), 0.1 ml of the broth culture, containing about 10^7 organisms per ml, was added, and the mixture was poured as an overlay over the chloroform-treated agar plates. After the overlay had gelled, the plates were incubated for 18 h at 37°C. The pyocin types were determined on the basis of inhibition of the indicator strains. Results were recorded in the same manner as described by Govan (20).

The pyocin type pattern was expressed as an octal code, similar to that used by the API 20E system (Analytab Products, Plainview, N.Y.). For example, if 14 indicator strains were used, four groups of 3 strains and a fifth group of 2 strains were arranged such that positive reactions by all 14 strains would yield an octal number of 77773, if positive reactions were scored from left to right with values of 1, 2, and 4. Negative reactions were assigned a value of 0; thus, untypable isolates produced an octal number of 00000.

Selection of the new pyocin typing set. Three critical factors were analyzed statistically: (i) the reproducibility of pyocin patterns generated by indicator strains, (ii) the ability of different sets or groups of indicator strains to discriminate among different strains of *P. aeruginosa*, and (iii) of less importance, the percentage of typable isolates. The first two factors were expected to affect one another.

Of the 416 cultures available for study from epidemiologic situations, 250 were selected randomly for study to develop a set of indicator strains for pyocin typing. The remaining 166 cultures were reserved to test the validity of the selected set of indicator strains by using a chi-square test to detect any differences between results with these two groups of isolates.

The reproducibility for an individual indicator strain was obtained by dividing the number of cultures in which repeated tests yielded exactly the same results by the total number of cultures tested against that indicator strain. Reproducibility values were obtained for individual indicator strains tested six times on the same day and for indicator strains tested on two different days. The time intervals between these duplicate tests ranged from a few days to as much as a year, although 90% of the test strains had duplicate tests performed within a mean of 45 days. The reproducibility of a typing set of 14 indicator strains was calculated for identity of repeated tests (i.e., no differences), for one reaction difference, and for two reactions difference in the repeat result. Both calculations involved dividing by the number of strains tested and multiplying by 100 to obtain percentage. Quality control cultures were included in each set of typing plates.

The VARCLUS procedure of SAS (19) and the P1M procedure of BMDP (13) were both applied with an IBM 3083 computer (IBM Corp., Armonk, N.Y.) to obtain the set of pyocin typing indicator strains with the widest diversity of reactions and thus the maximal ability to discriminate among different strains. The discriminatory ability of the typing set was defined as the number of pyocin types that represented 50% of the isolates tested. For reasons of convenience, we chose to use 14 indicator strains; thus, 14 clusters were obtained from the entire set of 31 indicator strains under investigation. The indicator strain with the best reproducibility on different days within each cluster was then selected. This procedure was found to produce certain clusters consisting of only one indicator strain of relatively poor reproducibility, so a modification was made by eliminating the indicator strain with the poorest reproducibility from the entire set. Cluster analysis was reapplied, and the best set of 14 indicator strains was selected from the 30 remaining indicator strains. This process was repeated several times; each time, the least reproducible of the remaining indicator strains under consideration was removed.

Cluster analysis was performed by calculating a measure of similarity and then applying a criterion for linking or combining clusters. The measure of similarity chosen was the value of the correlation. The linkage rules were: (i) single linkage, complete linkage, or average linkage in BMDP and (ii) maximizing the variation accounted for either by the first principle component or centroid component of each cluster in SAS.

The results from the pyocin typing experiments were analyzed statistically to test the disciminatory properties of the various indicator strains and to determine the reproducibility of results on given indicator strains both between test days and within any test day.

RESULTS

Reproducibility of the indicator strains. The same-day reproducibility of the 31 indicator strains was tested with 49 *P. aeruginosa* isolates which were pyocin typed six times on the same day (Table 1). Of the 31 indicator strains, 19 (61%) demonstrated same-day reproducibility greater than 95%. The Govan strains ranged from 79.6 to 100% reproducibility, with a mean of 92.9% for the same-day tests. The Farmer strains ranged from 83.7 to 100%, with a mean of 95.4% reproducibility. Different-day reproducibility results with a random subsample of 114 *P. aeruginosa* strains that were typed on two different days are also shown in Table 1. Of the 31 indicator strains, 14 (45%) demonstrated different-day reproducibility greater than 95%. The Govan and Farmer strains showed nearly identical average reproducibilities when tested on different days: 93.6% (range: 87.7 to 98.2%)

and 93.9% (range: 87.7 to 99.1%), respectively. Several indicator strains showed lower reproducibilities for same-day results than with results obtained on different days.

Selection and analysis of the new typing set. All measures of similarity and rules for linkage yielded similar results when indicator strain cluster analyses were produced by the VARCLUS procedure of SAS and the P1M program of BMDP. The results in Table 2 were generated by VARCLUS by using the value of the correlation as the measure of similarity and maximizing the variation accounted for by the first principle component of each cluster as the linkage rule.

Some of the results obtained when different clusters were analyzed as just described are illustrated in Table 2. Each set was designed to have 14 indicator strains; thus 14 clusters were analyzed and the least reproducible strains were discarded. The cluster analyses were performed 10 times, resulting in indicator strain sets *a* to *j*. The interplay of the three variables tested—percent typable, discrimination, and reproducibility—is shown in Table 2. All 10 of the G-F indicator strain sets typed 99.5% of the 114 cultures used, so the choice of indicator strain set was made on the grounds of discrimination and reproducibility achieved. The greatest discrimination was obtained when all 31 indicator strains (G-F set *a*) were included in the cluster analysis to select the best 14, but the poor level of reproducibility (51.7%) for no pattern differences was not acceptable. We arbitrarily established a minimum of 60% reproducibility (for no pattern differences) which would be acceptable for an indicator set; thus, only G-F sets *f* through *j* received further consideration. Of these, we considered sets *i* and *j* to have inadequate discriminatory ability. Of the remaining three sets (*f*, *g*, and *h*), set *f* had the highest discriminatory ability but the lowest reproducibility, and set *h* had the lowest discriminatory ability and the highest reproducibility. We elected, therefore, to use set *g*, which combined both high discriminatory ability and high reproducibility.

Among the original 31 indicator strains tested, 6 had been discarded to arrive at set *g* (Table 2). The 25 indicator strains remaining and the individual clusters into which they were arranged are shown in Table 3. The individual strains which were then selected for the final 14 to compose the typing set are indicated by asterisks. These were selected on the basis of their reproducibility in tests on different days (see Table 1). When two indicator strains had the same degree of reproducibility on different-day tests, the choice was made on the basis of reproducibility on the same day or on the basis of ease of reading reactions with those particular strains. The 14 chosen indicator strains were then arranged in the following order for the typing set: Govan 1, 4, 5, 6, B, C; Farmer ALA 1, 3, 4, 6, 11, 12, 14, and 15. This new set was designated G-F because it is a combination of indicator strains from the Govan and Farmer sets.

Typing pattern frequencies. The pyocin typing patterns of the 250 strains used for developing the typing set were compared with the patterns of 166 other *P. aeruginosa* strains by using a chi-square test to determine whether any significant difference existed between these two groups of cultures. The frequencies of the pyocin patterns were similar (Table 4). It is interesting that only one indicator strain reaction separates the two most common pyocin types. The statistical values indicate that there is no significant difference in the results obtained with the two sets of cultures, and thus the results obtained with the 250 strains used to develop the G-F indicator set are applicable to other cultures.

Typing of mucoid strains. Ten mucoid strains of *P. aeruginosa*, consisting of six isolates from sputum and four isolates from urine, were pyocin typed by using the Govan and G-F indicator strain sets. Nine strains produced clear typing patterns and one strain was untypable (results not shown).

TABLE 1. Reproducibility (%) of indicator strains

Group and strain	Reproducibility (%) of indicator strain on:	
	Same date[a]	Different date[b]
Govan		
1	97.9	97.4
2	95.9	96.5
3	95.9	96.5
4	95.9	95.6
5	95.9	95.6
6	93.9	92.1
7	91.8	97.4
8	89.9	90.4
A	87.7	89.5
B	100	98.2
C	85.7	90.4
D	79.6	87.7
E	98.0	89.5
Farmer		
ALA 1	83.7	93.0
ALA 2	100	89.5
ALA 3	97.9	93.9
ALA 4	97.9	97.4
ALA 5	87.7	90.4
ALA 6	95.9	93.0
ALA 7	97.9	93.0
ALA 8	93.9	89.5
ALA 9	95.9	94.7
ALA 10	95.9	89.5
ALA 11	95.9	99.1
ALA 12	100	98.2
ALA 13	100	98.2
ALA 14	93.9	96.5
ALA 15	91.8	93.9
ALA 16	97.9	87.7
ALA 17	93.9	96.5
ALA 18	97.9	95.6

[a] 49 strains typed six times on one date.
[b] Random subsample of 114 strains typed on two different dates.

DISCUSSION

Our evaluation confirmed that the revised pyocin typing method of Fyfe et al. (17) is much improved in comparison with the older cross-streaking technique of Gillies and Govan (18). The revised method allows for typing of multiple isolates on the same set of typing plates. This method also has the advantage over the cross-streaking and broth methods (16, 25) of reducing the time required to obtain typing results by 24 h and of the ability to type mucoid strains of *P. aeruginosa*. Researchers who already perform pyocin typing by the cross-streaking technique need no additional equipment other than a multipoint inoculator to perform the revised method.

One would normally expect the reproducibilities of individual indicator strains tested on the same day to be higher than when tested on different days. However, several indicator strains showed lower reproducibility among replicate tests on the same day. Two possible explanations for this result are: (i) two different sets of producer strains were used to determine the same-day and different-day reproducibilities and (ii) the criterion for same-day reproducibility is

TABLE 2. Discriminatory ability and reproducibility of Govan, Farmer, and G-F indicator strain sets[a]

Indicator strain set	No. of strains in set	No. of strains discarded[b]	% Typable	Discriminatory ability[c]	Pattern difference					
					0		1		2	
					Value[d]	% Reproducible	Value	% Reproducible	Value	% Reproducible
Govan (1–8, A–E)	13	0	98.8	7	66	57.9	28	82.5	7	88.6
Farmer (ALA 1–18)	18	0	99.3	8	62	54.4	21	72.8	15	86.0
G-F										
a	14	0	99.5	15	59	51.7	34	81.6	12	92.1
b	14	1	99.5	10	62	54.4	31	81.6	10	90.3
c	14	2	99.5	9	62	54.4	30	80.7	10	89.5
d	14	3	99.5	11	66	57.9	24	78.9	11	88.6
e	14	4	99.5	11	66	57.9	24	78.9	11	88.6
f	14	5	99.5	13	69	60.5	22	79.8	13	91.2
g	14	6	99.5	10	71	62.3	24	83.3	9	91.2
h	14	7	99.5	9	73	64.0	23	84.2	12	94.7
i	14	8	99.5	5	76	66.7	24	87.7	7	93.9
j	14	9	99.5	4	76	66.7	24	87.7	8	94.7

[a] Random subsample of 114 isolates.
[b] Number of indicator strains deleted because of poor reproducibility before cluster analysis was run.
[c] Number of pyocin types that represent 50% of isolates.
[d] For explanation of how value was derived, see Materials and Methods.

much stricter, in that the indicator strain reaction must be identical six times instead of two times, as required by different-day reproducibility tests.

Since some outbreaks and epidemiological investigations take place over extended periods of time, it is not always possible to type a collection of isolates at one time. For this reason we did not adhere to a strict timetable when conducting different-day reproducibility studies. A stable pyocin type pattern is necessary to obtain consistent results over various periods of time. Changes in inhibition patterns can be caused by the indicator strain (26), producer strain (20, 26), growth conditions (4, 8), or a combination of these or other factors. Although it was not possible to differentiate absolutely between these factors, certain indicator strains, particularly Govan A, C, and D and Farmer 2, 5, 8, 10, and 16, were consistently responsible for the vast majority of fluctuating patterns. In most cases, only one indicator strain exhibited fluctuation, as is shown in Table 2 under the column labeled "Pattern difference: 1." On several occasions, collections of cultures that were judged to be epidemiologically related exhibited changes in pyocin type patterns. However, even after these changes in pyocin type, the cultures still exhibited the same relatedness because the changes occurred uniformly in all of the related cultures. One would not expect several of the producer strains to change simultaneously, so this phenomenon seems to indicate changes taking place in the indicator strains. A few cultures showed multiple and inconsistent pattern changes over as many as 10 typings. Stable pyocin typing patterns could not be achieved with these cultures. Here the changes apparently occurred in the producer strains. We do not know at this time whether the nature of our cultures influenced this, i.e., whether freshly isolated cultures of *P. aeruginosa* might act differently than did our stock cultures. This will be investigated later.

Our percentage of typable strains with all three sets of indicator strains (Govan, Farmer, and G-F) either was comparable to or exceeded that observed by others (10, 14, 20). The new G-F indicator set was more discriminating and slightly more reproducible than the two sets of indicator strains from which it was formed. The discriminatory potential of the G-F set is 42.8% greater than that of the Gillies and Govan set.

One of the major problems we faced when attempting to develop an alternative set of indicator strains was that of selecting strains which were easy to interpret and were reproducible, without sacrificing discrimination. The indicator strains which were difficult to read were more likely to fare poorly in reproducibility studies. Unfortunately, the indicator strains that were the most difficult to interpret and least reproducible were among the most discriminating.

TABLE 3. Typing set cluster for G-F indicator strain set *g*

Cluster no.	Members of cluster[a]
1	ALA 12*
	ALA 13
	ALA 17
2	Govan 4*
	Govan 8
	ALA 2
	ALA 5
	ALA 18
3	Govan 1*
	Govan 2
	Govan 3
	Govan 7
4	ALA 3*
	ALA 9
5	Govan B*
6	ALA 6*
7	ALA 14*
8	ALA 15*
9	ALA 1*
10	Govan C*
11	Govan 6*
12	ALA 4*
13	ALA 7
	ALA 11*
14	Govan 5*

[a] Asterisk (*) denotes indicator strain chosen for new typing set.

TABLE 4. Comparison of pyocin typing patterns with G-F indicator strain set[a]

Pyocin type octal code	No. of isolates (% of total) in:	
	Group 1[b]	Group 2[c]
77773	28 (11.2)	17 (10.2)
73773	23 (9.2)	16 (9.6)
54373	18 (7.2)	11 (6.6)
76773	13 (5.2)	5 (3.0)
56773	10 (4.0)	3 (1.8)
74763	8 (3.2)	5 (3.0)
74773	8 (3.2)	5 (3.0)
76763	9 (3.6)	4 (2.4)
75773	4 (1.6)	7 (4.2)
73673	7 (2.8)	1 (0.6)
30203	3 (1.2)	5 (3.0)
73473	3 (1.2)	5 (3.0)
42473	3 (1.2)	4 (2.4)
All others	113 (45.2)	78 (47.2)

[a] $\chi^2 = 4.15$ ($P = 0.843$) comparing groups 1 and 2. Patterns 75773 to 42473 were included in "All others" for the χ^2 test.
[b] 250 isolates used to select G-F typing set.
[c] 166 isolates used for challenge (to test the validity of the new typing set).

There appears to be an inverse relationship between reproducibility and the ability to discriminate between test isolates when no more than one pattern difference is allowed.

The new G-F typing set contains only one indicator strain, Govan C, which still continues to present problems in interpretation and reproducibility. This indicator strain is responsible for separating the two most common pyocin types, and according to Table 1 has one of the lowest same-day reproducibilities (85.7%). It is our intention that a replacement will be sought for the Govan C strain in the near future.

An approach similar to that of Bergan (4) and Grajewski et al. (22), who applied numerical taxonomic techniques to the selection of phages for typing *P. aeruginosa*, and *Campylobacter jejuni* and *Campylobacter coli*, respectively, was used in this study to select the new set of indicator strains. The two computer programs used in this study, the VARCLUS procedure of SAS and the P1M program of BMDP, both produced similar clusters of indicator strains. For example, the Farmer ALA indicator strain set contains two members from the Govan typing set: ALA 5 was Govan indicator strain 4, and ALA 16 was Govan indicator strain B. Regardless of the computer program used, these two pairs of indicator strains always clustered the same. The VARCLUS procedure was chosen to make the final selection of indicator strains for the new typing set because of its simplicity. Use of numerical taxonomic techniques to form, modify, or increase a pyocin typing set is highly recommended. Cluster analysis provides a logical method for selecting indicator strains, since it considers the indicator strains collectively rather than individually.

We feel that the revised pyocin typing procedure is a reliable method for typing *P. aeruginosa*. The G-F indicator strain set may prove to be slightly more discriminating than the Gillies and Govan set, depending on the isolates tested. Even though ease of interpretation is a subjective judgment, the G-F indicator strain set, when used with our collection of 416 isolates, was easier to read than either of the other two sets.

Use of an octal code system has greatly simplified reporting pyocin typing results. Of all the current schemes for reporting patterns of inhibition of indicator strains (11, 15, 20, 28), we consider this method the easiest and most logical.

ACKNOWLEDGMENTS

We thank J. R. W. Govan and J. J. Farmer III for providing the *Pseudomonas* pyocin indicator strains. We also appreciate and gratefully acknowledge Anita K. Highsmith and Robert E. Weaver for providing us with the clinical and environmental *Pseudomonas* isolates.

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