IDENTIFICATION OF *PSEUDOMONAS AERUGINOSA* IN THE CLINICAL LABORATORY

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PLATES I AND II

THE literature concerning the identification of *Pseudomonas aeruginosa* is large and increasing (see Jessen, 1965; Stanier, Palleroni and Doudoroff, 1966). Because of this, it is difficult to choose a series of tests that, while technically simple and suitable for use in a hospital diagnostic laboratory, will yield the most useful information in the least time. The purpose of this paper is to report an assessment of some of the methods in a hospital laboratory, and to suggest a simple scheme of identification.

The tests were first applied to 100 pyocyanin-producing strains of *Ps. aeruginosa* from varied clinical sources. On the basis of the results of this exercise, a determinative scheme was drawn up and used in an epidemiological investigation of *Ps. aeruginosa* respiratory-tract infections in St Thomas's Hospital during 1965.

MATERIALS AND METHODS

Primary plating of all samples always included aerobic culture on blood-agar medium. Colonies grown on this medium were then subcultured in all other media used in the identification procedure. Unless otherwise stated, liquid media were dispensed in 3-4-ml amounts in screw-capped bijou bottles. The nutrient broth used was Oxoid no. 2. Except for tests for growth at 42°C, incubation was at 37°C.

Methods used in this investigation and described by Cowan and Steel (1965) or Cruickshank (1965) are not described in detail here.

Observation of colonial form. Cultures on blood-agar plates were examined after overnight incubation for colonial forms characteristic of *Ps. aeruginosa* (Wahba and Darrell, 1965) and for the characteristic odour of the organism.

Observation of pigment production: (a) *Fluorescence.* Colonies on blood agar were examined in a dark chamber by ultraviolet light (Lowbury, Lilly and Wilkins, 1962); the lamp was Model A409 made by P. W. Allen and Co., 253 Liverpool Rd, London, N. 1. Some strains, proved to be *Ps. aeruginosa* but not fluorescent on blood agar, were re-examined on Pseudomonas Agar F (Difco) enhancement medium. (b) *Pyocyanin and pyorubin.* Pseudomonas Agar P (Difco) was dispensed as slopes in 25-ml screw-capped bottles. Cultures on these slopes were incubated with the caps loose and examined daily for blue or red pigment.

Oxidase test. A mixture of equal parts of 1 per cent. aqueous dimethyl-*p*-phenylenediamine and 1 per cent. α -naphthol in 95 per cent. ethanol was poured on to colonies on blood agar. Oxidase-positive colonies became dark blue within a few seconds (Gaby and Hadley, 1957).

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Test for oxidative metabolism of glucose. The medium of Hugh and Leifson (1953), as described by Cowan and Steel (p. 107), was dispensed in 2-ml amounts in screw-capped bottles; the aerobic test bottles were incubated with the caps loose.

Test for arginine dihydrolase. The medium had the following composition: Difco Bactopeptone, 1 g; sodium chloride, 5 g; dipotassium phosphate, 0.3 g; agar, 3 g; phenol red, 0.01 g; L-arginine hydrochloride, 10 g; distilled water to 1 litre; pH 7.2. Two bottles were inoculated with each strain and one culture was made anaerobic by the addition of a layer of melted Vaseline. Arginine dihydrolase activity was detected by a change in colour of the indicator to red in both bottles (Thornley, 1960).

Test for oxidation of gluconate. The modification of the method of Haynes (1951) described by Cruickshank (p. 831) was used, with 3-ml volumes of medium incubated for 3 days. After incubation, the culture was swirled round to detect slime; half a "Clinitest" tablet (Ames) was then added and the medium observed on the bench for the formation of a precipitate of cuprous oxide.

Test for growth at 42°C. The test strain was inoculated into nutrient broth warmed to 42°C and then incubated at 42°C in a waterbath. If growth occurred after overnight incubation, two more serial subcultures were attempted in nutrient broth at 42°C (Haynes and Rhodes, 1962).

Test for growth in 6 per cent. sodium chloride in nutrient broth. To 1 litre of distilled water were added 55 g NaCl and 25 g dehydrated nutrient broth; the mixture was dispensed in bottles and autoclaved. The test strain was inoculated into the medium which was incubated overnight and then examined for growth (Rhodes, 1959).

Test for growth in 0.03 per cent. cetrimide in nutrient broth. A 30-ml volume of a 1 per cent. cetrimide solution was added to 970 ml of nutrient broth, dispensed into bottles, and autoclaved for 15 min. at 15 lb. per sq. in. (121°C). The test strain was inoculated and the medium examined for growth after 24 and 48 hours' incubation. This was an adaptation of the method used by Lowbury and Collins (1955) for the isolation of *Ps. aeruginosa*.

Test for growth in 1 per cent. triphenyl tetrazolium chloride (T.T.C.) in nutrient broth. A 10-g amount of T.T.C. was added to 1 litre of nutrient broth, dispensed into bottles, and autoclaved for 15 min. at 121°C. The test strain was inoculated and the medium examined for the development of a red precipitate after 24 and 48 hours' incubation (Selenka, 1958).

Test for reduction of nitrate to gaseous nitrogen. The method is described by Cowan and Steel (p. 161).

Test for hydrolysis of acetamide. The basic medium was that of Christensen (1946) as described by Cowan and Steel (p. 125) but without glucose; acetamide, sterilised by Seitz filtration, was incorporated at a final concentration of 1 per cent. in the test medium but not in the otherwise identical control medium. The test organism was cultured in 2 bottles of the medium, one with and the other without acetamide; these were incubated and examined daily for the change to red of the indicator in the bottle containing acetamide but not in the control, which indicated that hydrolysis had taken place (Bühlmann, Vischer and Bruhin, 1961).

Test for liquefaction of gelatin. Kohn's (1953) method as described by Cruickshank (p. 823) was used.

RESULTS

Studies with 100 pyocyanin-producing strains of Ps. aeruginosa

All these strains were identified by their ability to produce pyocyanin on Pseudomonas Agar P, usually within 24 hr, but occasionally after 48 hr or more. All the colonial forms reported by Wahba and Darrell, with the exception of the gelatinous one, were encountered; they are illustrated in figs. 1-6. Most of the strains were fluorescent on blood agar; this fluorescence was most marked in young cultures, and in strains having coliform-like colonies. On blood agar the blue colour of a positive cytochrome oxidase test was often not

apparent until up to 60 s after the application of the reagent. The times by which positive results were obtained in the other tests are listed in table I.

In view of what was known of the specificity of these tests from the work of the authors who described them, and the rapidity with which results were obtained in this study, the determinative scheme in table II was drawn up and applied in the epidemiological investigation.

TABLE I
Speed of obtaining positive results in ten tests on 100 pyocyanin-producing strains of Ps. aeruginosa

Test	Number of strains giving positive results on day			
	1	2	3	4 or later
Glucose oxidation	100	...	...	...
Arginine hydrolysis . . .	100	...	...	...
Gluconate oxidation . . .	*	*	100	...
Growth at 42°C	*	*	100	...
Growth in 6 per cent. NaCl . . .	95	5	...	...
Growth in cetrimide	96	4	...	...
Growth in T.T.C.	97	3	...	...
Reduction of nitrate	*	*	100	...
Hydrolysis of acetamide . . .	18	32	50	...
Liquefaction of gelatin . . .	81	11	3	5

* Not assessed on these days, as in each case the method requires incubation for 3 days.

Application of the determinative scheme

During the survey of respiratory-tract infections, the scheme was applied in the examination of more than 2000 samples. Apart from repeated isolations from the same patient or the same site, 127 separate strains of *Ps. aeruginosa* were isolated from 71 patients and their environments.

Colonial form. Table III shows the incidence of the various colonial forms. Colony type 1 strains were immediately recognisable as *Ps. aeruginosa* and their identity was confirmed by testing for pyocyanin production. Colony type 2 strains were particularly common in samples from the environment. Rough, rugose, mucoid, and dwarf colonies were all rare. The dwarf colony type was investigated as a possible *Haemophilus influenzae* before its true nature was appreciated.

Fluorescence. Table IV gives the results of examination for fluorescence on blood agar and on enhancement medium. Sometimes fluorescence was pronounced in colonies shown subsequently *not* to be *Ps. aeruginosa*; the fluorescence resulted from absorption of fluorescein from nearby *Pseudomonas* colonies. False positive results, presumably due to accumulation of tetracycline in resistant colonies, were also seen in colonies growing around tetracycline disks in direct sensitivity tests.

Pyocyanin production. Table IV shows the results of examination for

TABLE II
Key for the identification of *Ps. aeruginosa*

Test no.	Description	Proceed to test no.	Conclusion
1	Colonies typical (type 1) . . . Not as above	6 2	
2	Fluorescent Not as above	6 3	
3	Oxidase-positive Not as above	4 ...	not <i>Pseudomonas</i>
4	Gram-negative bacilli * . . . Not as above	5 ...	not <i>Pseudomonas</i>
5	Oxidative metabolism of glucose . Not as above	6 ...	not <i>Pseudomonas</i>
6	Pyocyanin produced Not as above	... 7†	<i>Ps. aeruginosa</i>
7	Arginine dihydrolase activity . Not as above	8 ...	not <i>Pseudomonas</i>
8	Gluconate oxidised Not as above	9 ...	not <i>Ps. aeruginosa</i>
9	Growth at 42°C in serial cultures . Not as above		<i>Ps. aeruginosa</i> <i>Pseudomonas</i> species, not <i>aeruginosa</i>

* In many cases, the organism will already have been shown to be a Gram-negative bacillus.

† If an organism with a typical colonial form fails to produce pyocyanin, return to test 2 and proceed with tests 3, 4, 5 and then 7, 8 and 9. Identification of an organism as an apyocyaninogenic strain of *Ps. aeruginosa* should be confirmed by demonstrating its ability to grow in the presence of T.T.C., and to reduce nitrate to nitrogen.

TABLE III
Colonial forms of 127 strains of *Ps. aeruginosa* isolated during an epidemiological study of respiratory tract infections

Colony type	Description	Number of strains	Percentage frequency
1	Typical, with or without other types .	90	71
2	Coliform-like	27	21
3 or 4	Rough or rugose	8	6.5
5	Mucoid	2	1.5
6	Dwarf	1*	0.75

* The dwarf variant arose from one of the mucoid types.

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FIG. 1.—Colony type 1, typical.
24 hr at 37°C. $\times 5$.

FIG. 2.—Colony type 2, coli-form-like. 24 hr at 37°C.
 $\times 5$.

FIG. 3.—Colony type 3, rough.
24 hr at 37°C. $\times 5$.

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FIG. 4.—Colony type 4, rugose.
24 hr at 37°C. $\times 5$.

FIG. 5.—Colony type 5, mucoid.
24 hr at 37°C. $\times 5$.

FIG. 6.—Colony type 6, dwarf.
48 hr at 37°C. $\times 5$.

pyocyanin production on *Pseudomonas* Agar P. Production of pyorubin in strains producing little pyocyanin was also enhanced by this medium. One strain produced a brown "melanin" pigment (De Ley, 1964). Only one strain produced no pigment of any kind.

Other tests. In all other tests these 127 strains behaved as the 100 pyocyaninogenic strains listed in table I.

TABLE IV

Pigment production by 127 strains of Ps. aeruginosa isolated during an epidemiological study of respiratory tract infections

Substance and test data relating production to cultural conditions	Number of strains	Percentage frequency
<i>Fluorescin</i>		
Positive on blood agar	115	90
Positive only on enhancement medium	5	4
Negative	7	6
<i>Pyocyanin</i> *		
Positive in 24 hr	113	89
Positive in > 24 hr	9	7
Negative	5	4
<i>Pyorubin</i> *		
Dominant	7	6
Slight or absent	120	94

* Strains cultured on *Pseudomonas* Agar P (Difco).

DISCUSSION

As the methods tested with the 100 known strains of *Ps. aeruginosa* were chosen on the basis of criteria that would determine their usefulness in a routine laboratory—simplicity, reliability, and rapidity—many properties, some of prime importance in taxonomy, were ignored. From the point of view of simplicity, an important step in tests involving liquid medium was the use of small volumes of medium in screw-capped bottles. This eliminated the problem of bulk, and also allowed storage of the medium for long periods without dehydration. Selective media were not used in the isolation of the organism, as selective culture is not the method by which *Ps. aeruginosa* is usually isolated in routine diagnostic work.

In the epidemiological survey, colonies were selected from primary cultures on the basis of three criteria: (1) recognition of the typical colonial form—colony type 1—which led to immediate identification in 70 per cent. of cases; (2) fluorescence, in this work highly specific for *Ps. aeruginosa* as few of the other fluorescent pseudomonas species were encountered; (3) oxidase activity. Although the cytochrome oxidase test did not, in our hands, have the specificity for *Ps. aeruginosa* originally claimed for it as applied to isolations from clinical

material (Gaby and Hadley, 1957; Gaby and Free, 1958), it was important in excluding many colonies from further investigation. Determination of these three properties involved a minimum of work and led to the identification of most strains, which required only the further demonstration of pyocyanin production for confirmation.

Stages 4 and 5 in the scheme—Gram staining, and the Hugh and Leifson test for oxidation or fermentation of glucose—were useful in eliminating most of the oxidase-positive colonies that did not belong to the genus *Pseudomonas*. The Gram stain was particularly useful *at this stage* in the examination of sputum, in eliminating *Neisseria*, and, in the study of environmental samples, in eliminating *Bacillus* species. In many situations in clinical bacteriology the Gram stain is the first investigation to be done in the process of identification, but in the examination of mixed populations, especially of environmental origin, with the aim of identifying *Pseudomonas* only, it is probably best deferred.

It was not easy to choose the medium for the most important test in the scheme—that for enhancement of pyocyanin production. Very many such media have been described (Gessard, 1892, 1920; Georgia and Poe, 1931; Seleen and Stark, 1943; Burton, Campbell and Eagles, 1948; King, Ward and Raney, 1954; Martineau and Forget, 1958; Rosenfeld and Appel, 1963; Wahba and Darrell, 1965). Difco *Pseudomonas* Agar P was eventually chosen on the grounds of commercial availability; the finding that only 4 per cent. of strains failed to produce pyocyanin on this medium compares well with the results that Wahba and Darrell obtained with their medium.

Stages 7 and 8 in the key define the genus *Pseudomonas*, but the earlier application of these criteria in the system would not have done so because other organisms can have arginine dihydrolase activity and oxidise gluconate (Brown and Lowbury, 1965). In our work, few organisms were studied beyond these two steps.

Only five strains of these few grew consistently at 42°C and were therefore identified as apyocyaninogenic *Ps. aeruginosa*. In such cases, other confirmatory tests should be applied; the differentiation from other pseudomonas species by this scheme rests entirely on the criteria of growth at 42°C and the production of slime in the gluconate medium. All the methods listed in table I should give positive results if the organism is *Ps. aeruginosa*, but other pseudomonas species may also do so. Growth on T.T.C., and reduction of nitrate to nitrogen appear to characterise the species *Ps. aeruginosa* (Brown and Lowbury; Wahba and Darrell), but growth on cetrinide, hydrolysis of acetamide, and growth in 6 per cent. sodium chloride are properties shared by several members of the genus (Brown and Lowbury; Jessen, 1965). Experience with apyocyaninogenic strains is limited. Wahba and Darrell reported on the investigation of 34 strains that failed to produce pyocyanin on enhancement medium; all gave reactions similar to those of pigmented strains. Other pseudomonas organisms were found to produce slime rarely in gluconate medium, and to fail to grow, in most cases, on media containing T.T.C. or cadmium sulphate. Wahba and Darrell also found collagenase production to be a useful differential

property: most strains of *Ps. aeruginosa* produced it whilst other pseudomonas strains did not.

In practice, the use of the key suggested in table II led to the complete identification of *Ps. aeruginosa* by the demonstration of pyocyanin production, which is unique to the species, within 24 hr of primary isolation in most instances. Almost all the remaining strains were positively identified within a further 24 hr.

SUMMARY

By testing some of the many methods that have been described for the identification of *Ps. aeruginosa* with a series of typical strains an identification scheme was evolved and subsequently applied in an epidemiological investigation of *Ps. aeruginosa* respiratory tract infections. Its use led to the rapid identification of typical strains, but even atypical strains were usually identified within 48 hr of their isolation. The scheme has been evolved with the needs of the routine medical diagnostic laboratory in mind.

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REFERENCES

- BROWN, V. I., AND LOWBURY, E. J. L. 1965. *J. Clin. Path.*, **18**, 752.
 BÜHLMANN, X., VISCHER, W. A., AND BRUHIN, H. 1961. *J. Bact.*, **82**, 787.
 BURTON, M. O., CAMPBELL, J. J. R., AND EAGLES, B. A. 1948. *Canad. J. Res.*, **26C**, 15.
 CHRISTENSEN, W. B. 1946. *J. Bact.*, **52**, 461.
 COWAN, S. T., AND STEEL, K. J. 1965. Manual for the identification of medical bacteria, Cambridge.
 CRUICKSHANK, R. 1965. Medical microbiology, 11th ed., Edinburgh.
 DE LEY, J. 1964. *A. Rev. Microbiol.*, **18**, 17.
 GABY, W. L., AND FREE, E. 1958. *J. Bact.*, **76**, 442.
 GABY, W. L., AND HADLEY, C. 1957. *Ibid.*, **74**, 356.
 GEORGIA, F. R., AND POE, C. F. 1931. *Ibid.*, **22**, 349.
 GESSARD, C. 1892. *Annls Inst. Pasteur, Paris*, **6**, 801.
 " 1920. *Ibid.*, **34**, 88.
 HAYNES, W. C. 1951. *J. Gen. Microbiol.*, **5**, 939.
 HAYNES, W. C., AND RHODES, LEONORA J. 1962. *J. Bact.*, **84**, 1080.
 HUGH, R., AND LEIFSON, E. 1953. *Ibid.*, **66**, 24.
 JESSEN, O. 1965. *Pseudomonas aeruginosa* and other green-fluorescent pseudomonads, Copenhagen.
 KING, ELIZABETH O., WARD, MARTHA K., AND RANEY, D. E. 1954. *J. Lab. Clin. Med.*, **44**, 301.
 KOHN, J. 1953. *J. Clin. Path.*, **6**, 249.
 LOWBURY, E. J. L., AND COLLINS, A. G. 1955. *Ibid.*, **8**, 47.
 LOWBURY, E. J. L., LILLY, H. A., AND WILKINS, M. D. 1962. *Ibid.*, **15**, 339.

- MARTINEAU, B., AND FORGET, A. . . . 1958. *J. Bact.*, **76**, 118.
RHODES, MURIEL E. 1959. *J. Gen. Microbiol.*, **21**, 221.
ROSENFELD, J., AND APPEL, G. . . . 1963. *Amer. J. Med. Technol.*, **29**, 189.
SELEEN, W. A., AND STARK, C. N. . . . 1943. *J. Bact.*, **46**, 491.
SELENKA, F. 1958. *Arch. Hyg. Bakt.*, **142**, 569.
STANIER, R. Y., PALLERONI, N. J., AND DOUDOROFF, M. . . . 1966. *J. Gen. Microbiol.*, **43**, 159.
THORNLEY, M. J. 1960. *J. Appl. Bact.*, **23**, 37.
WAHBA, A. H., AND DARRELL, J. H. . . . 1965. *J. Gen. Microbiol.*, **38**, 329.