Identification of *Pseudomonas pseudomallei* in the clinical laboratory

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SUMMARY Ninety-one strains of *Pseudomonas pseudomallei* were tested in the API 20E system and in equivalent conventional tests. Except for utilisation of citrate there was good correlation between API and conventional tests. Seven-digit profiles were constructed from each strain after 48 hours' incubation, and numerical codes 2 006 727, 2 206 706, 2 206 707, and 2 206 727 accounted for 77% of strains tested. API 20E, in combination with tests for heat stability of alkaline phosphatase, resistance to colistin and gentamicin, oxidative attack only of glucose, and acid from maltose, was found to provide a simple method for positive identification of all strains of this organism in two days.

Melioidosis is a rare, infectious disease with primary endemic foci in South-east Asia, northern Australia, and Central America. In the acute form, it is often fulminating and fatal, while chronic forms of the disease have been reported in veterans of south-east Asian conflicts and in visitors and residents of endemic areas (Howe *et al.*, 1971). Clinical diagnosis is made by isolation of the causative bacterium, *Pseudomonas pseudomallei*, supported by the demonstration of high specific antibody. Identification of an isolate as *Ps. pseudomallei* is, however, difficult due to sharing of physical and biochemical properties with other bacteria and because of the infrequency with which it is encountered, while diagnosis is often delayed since the isolate is frequently submitted to a reference laboratory for identification (Zierdt and Marsh, 1971). Final confirmation is made by serology using specific antiserum against *Ps. pseudomallei*, but this is rarely a routine procedure in clinical laboratories as specific antigen and reference antisera for this uncommon disease are not easily obtained. A simple method for positive identification of *Ps. pseudomallei* that is within the scope of the small diagnostic laboratory seems to be an obvious requirement.

The commercially produced kit system, API 20E (Analytab Products Inc, New York, USA), is currently used in many laboratories for the identification of Enterobacteriaceae and other Gram-negative bacteria. The kit consists of 20 plastic cupules containing dehydrated media which are

inoculated with a suspension of the test organism and incubated at 37°C from 18-24 hours to 36-48 hours, depending on the number of positive reactions. Twenty-one tests in this system are divided into seven groups of three, and each test is assigned a number. By this method a seven-digit profile of reactions of the test organism is determined, and the profile number is matched against numbers in a computerised Analytical Profile Index to identify the organism. However, strains of *Ps. pseudomallei* are not included in the range of organisms in the API data base, and no profile numbers for *Ps. pseudomallei* exist in the Index at this time (August, 1978).

Liu (1966) reported that alkaline phosphatases of *Ps. pseudomallei* and *Ps. aeruginosa* were heat resistant while those of other pseudomonads were heat labile. This test together with susceptibility to colistin and gentamicin, oxidation-fermentation reactions of glucose, and acid reaction from maltose were considered as valuable tests in the differentiation of *Ps. pseudomallei* from other Gram-negative bacteria. The purpose of this paper is to compare tests of the API system against conventional methods, construct seven-digit profiles for *Ps. pseudomallei*, and evaluate API 20E with the above additional tests as criteria for identification of *Ps. pseudomallei* from other oxidase-positive Gram-negative bacilli.

Material and methods

TEST ORGANISMS

Ninety-one strains of *Ps. pseudomallei* were available

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for testing: 37 strains were donated from the School of Public Health and Tropical Medicine, University of Sydney, 23 strains from the Animal Health Station, Townsville, 5 strains from the Australian Government Health Laboratory (AGHL), Rockhampton, and 26 strains from the AGHL, Townsville. Other organisms tested were: 18 strains of *Ps. aeruginosa*, 10 strains of *Ps. cepacia*, 9 strains of *Ps. stutzeri*, 8 strains of *Ps. fluorescens*, 3 strains of *Alcaligenes bronchisepticus*, 2 strains of *A. faecalis*, and 2 strains of *Flavobacterium* species. These strains were isolated by me at the AGHL, Townsville, and each organism was identified according to Cowan (1974).

CULTURE STUDIES

The API 20E kit tests were performed according to the manufacturer's instructions which include details on inoculation and test interpretation. API kits were inoculated with test strains of pseudomonads suspended in sterile normal saline and incubated for two days at 37°C, and reactions were noted after 24 and 48 hours. Reagents were added to the system after 48 hours' incubation. Seven-digit profiles from API reactions of each strain were formulated according to the manufacturer's instructions. A sample of profile construction is given in Table 1 for strain NCTC 8016.

Conventional tests were carried out with test strains of *Ps. pseudomallei* using the media and methods of Cowan (1974). Specific methods used

were: β galactosidase production (ONPG test), decarboxylase reactions (method 2), citrate utilisation (method 2), H₂S production (method 1), urease production (method 2), phenylpyruvic acid production (method 2), indole production (method 2), acetoin production (V-P test, method 1), gelatin liquefaction (method 5), acid from carbohydrates (open tubes of OF basal medium with final concentration of all carbohydrates 1 g/100 ml), cytochrome oxidase activity (method 1). Tests for acid production from amygdalin were not performed.

Heat stability of alkaline phosphatase, resistance to colistin and gentamicin, oxidation-fermentation reactions from glucose and acid reaction from maltose were performed on all test strains. Heat resistance of alkaline phosphatase was determined as described by Liu (1966). A heavy 1-ml suspension of each test organism from an overnight growth on agar medium containing 0.2% peptone and 0.05% yeast extract was made in 0.02 M Tris buffer (pH 8.0). The inoculum was divided into two tubes, and one tube was heated at 70°C for 20 minutes. Equal volumes of 0.1% *p*-nitrophenylphosphate were added to both heated and unheated tubes and incubated overnight at 37°C and colour reactions were noted. Alkaline phosphatase was considered to be heat resistant if a yellow colour was produced in both tubes, and heat labile if colour was formed only in the unheated tube.

Susceptibility to colistin and gentamicin was determined by the method of Bauer *et al.* (1966). Test strains were considered resistant if zone diameters were 8 mm or less for colistin and 12 mm or less for gentamicin. Oxidation-fermentation reactions from glucose were carried out as described by Hugh and Leifson (1953), while acid reaction from maltose was determined using the OF basal medium of Hugh and Leifson (1953) with 1 g of maltose/100 ml of medium in unsealed tubes.

Results

Individual tests of the API system correlated well with conventional methods, except for the test for utilisation of citrate (Table 2), with which 32 strains of *Ps. pseudomallei* gave false negative reactions and one strain gave a false positive reaction. After 24 hours' incubation, arginine dihydrolase, glucose, mannitol, inositol, sorbitol, and arabinose generally gave weak reactions in both systems, becoming strongly positive after 48 hours' incubation. Four strains failed to give an acid reaction with glucose in the API system but were positive after 48 hours' incubation in conventional media. Gelatin liquefaction occurred with all strains after 24 hours' incubation. Seven-digit profiles were constructed from each

Table 1 Construction of seven-digit profile for *Pseudomonas pseudomallei*, strain NCTC 8016

Value of test	Test	Positive reaction	Value assigned	Numerical code*
1	ONPG	—	0	
2	Arginine dihydrolase	+	2	2
4	Lysine decarboxylase	—	0	
1	Ornithine decarboxylase	—	0	
2	Citrate utilisation	+	2	2
4	H ₂ S production	—	0	
1	Urease production	—	0	
2	Tryptophan deamination	—	0	0
4	Indole production	—	0	
1	Acetoin production	—	0	
2	Gelatin liquefaction	+	2	6
4	Acid from glucose	+	4	
1	Acid from mannitol	+	1	
2	Acid from inositol	+	2	7
4	Acid from sorbitol	+	4	
1	Acid from rhamnose	—	0	
2	Acid from sucrose	—	0	0
4	Acid from melibiose	—	0	
1	Acid from amygdalin	+	1	
2	Acid from arabinose	+	2	7
4	Cytochrome oxidase activity	+	4	

*Profile is given by 2 206 707 and only one profile exists for this strain

Table 2 Comparison of tests of API system with conventional tests using 91 strains of *Pseudomonas pseudomallei*

Test	API system*	Conventional tests	No. of strains in disagreement with conventional tests
ONPG	0	0	0
Arginine dihydrolase	98	100	2
Lysine decarboxylase	0	0	0
Ornithine decarboxylase	0	0	0
Citrate utilisation	63	98	33
H ₂ S production	0	0	0
Urease production	0	0	0
Tryptophan deamination	0	0	0
Indole production	0	0	0
Acetoin production	0	0	0
Gelatin liquefaction	100	100	0
Acid from glucose	95	100	4
Acid from mannitol	100	100	0
Acid from inositol	100	98 (2)	0
Acid from sorbitol	100	99 (1)	0
Acid from rhamnose	0	0 (2)	2
Acid from sucrose	43	40 (3)	0
Acid from melibiose	0	2 (3)	4
Acid from amygdalin	60	NT	—
Acid from arabinose	93	96 (4)	6
Cytochrome oxidase activity	100	100	0

*Figures indicate percent positive at 48 hours; those in parentheses indicate percent positive after 48 hours
NT = not tested

pseudomonad strain, and the numerical codes are given in Table 3.

All strains of *Ps. pseudomallei* and *Ps. aeruginosa* produced alkaline phosphatases which were heat stable, while those from other bacteria tested were heat labile. One strain of *A. bronchisepticus* and two strains of *Flavobacterium* species showed no evidence of alkaline phosphatase production. All strains of *Ps. pseudomallei* were resistant to colistin and gentamicin, oxidatively attacked glucose, and gave acid reactions from maltose. Results of tests from all strains are summarised in Table 4.

Discussion

Except for the test for utilisation of citrate, there was no significant difference between results from conventional tests and those obtained with API 20E. Butler *et al.* (1975) and Holmes *et al.* (1977) also found disagreement with API citrate and the conventional test. Although in some cases reactions could be read after 24 hours' incubation, it was found necessary to reincubate the API strips for a further 24 hours to obtain satisfactory readings. Reactions with API 20E were relatively consistent, with profiles 2 006 727, 2 206 706, 2 206 707, and 2 206 727 accounting for 77% of all strains tested. Results of biochemical tests of strains of *Ps. pseudomallei* were in agreement with those of other workers

Table 3 Comparison of seven-digit profiles from *pseudomonads* tested with API 20E after 48 hours' incubation

Organism	Profile	No. of patterns
<i>Ps. pseudomallei</i> (91 strains)	0 006 727	1
	0 206 706	1
	2 006 704	1
	2 006 706	4
	2 006 707	5
	2 006 726	1
	2 006 727	22
	2 202 704	1
	2 202 706	3
	2 206 704	4
	2 206 706	21
	2 206 707	12
	2 206 727	15
<i>Ps. aeruginosa</i> (18 strains)	0 206 006	1
	2 002 004	1
	2 006 004	5
	2 206 004	9
	2 206 006	1
<i>Ps. cepacia</i> (10 strains)	0 004 004	3
	0 206 006	1
	1 204 004	1
	4 304 004	4
	5 304 004	1
<i>Ps. stutzeri</i> (9 strains)	0 000 004	1
	0 004 004	5
	0 004 104	3
<i>Ps. fluorescens</i> (8 strains)	0 204 004	2
	2 000 004	5
	2 200 004	1

except for urease production which Cowan (1974) classed as a variable reaction. Hugh and Gilardi (1974) reported 35% of 26 strains tested as urease positive but in this series all 91 strains of *Ps. pseudomallei* tested in both API and conventional medium were negative for urease production.

Heat resistance of alkaline phosphatase proved to be a valuable test for the differentiation of *Ps. aeruginosa* and *Ps. pseudomallei* from other pseudomonads, especially *Ps. stutzeri* and *Ps. cepacia*. These resemble *Ps. pseudomallei* biochemically and morphologically, producing dry and wrinkled colonies which are similar to the rugose form of *Ps. pseudomallei*. However, as Liu (1966) pointed out, alkaline phosphatases of many enteric bacilli are heat resistant, and this test is valuable only for differentiation of species within the genus *Pseudomonas*. Generally, *Ps. aeruginosa* is readily differentiated from *Ps. pseudomallei*; however, some nonpyocyanogenic strains which are resistant to gentamicin and colistin can pose a problem in identification, and effectively mimic *Ps. pseudomallei* (Zierdt and Marsh, 1971). *Ps. aeruginosa* can be eliminated by its failure to react with maltose to produce an acid reaction. The oxidation-fermentation reaction separates glucose oxidising bacteria from other fermentative and non-fermentative organisms. Hall and Manion (1973) found that approximately one-third

Table 4 Comparative value of tests for differentiation of *Pseudomonas pseudomallei* from other commonly isolated Gram-negative bacteria

Organism	No. of strains tested	Tests					
		Heat stable* alkaline phosphatase	Resistance* to:		Oxidation† of glucose	Fermentation† of glucose	Acid† from maltose
			Colistin	Gentamicin			
<i>Ps. pseudomallei</i>	91	100	100	100	100	0	100
<i>Ps. aeruginosa</i>	18	100	0	16	100	0	0
<i>Ps. cepacia</i>	10	0	80	90	100	0	100
<i>Ps. stutzeri</i>	9	0	0	0	100	0	100
<i>Ps. fluorescens</i>	8	0	0	0	100	0	75
<i>A. bronchisepticus</i>	3	0‡	0	0	0	0	0
<i>A. faecalis</i>	2	0	0	0	0	0	0
<i>Flavobacterium</i> sp	2	NR	0	0	0	0	0

*Percentage positive after overnight incubation

†Percentage positive after 48 hours' incubation

‡No reaction occurred with one strain

NR = no reaction

of 51 strains of *Ps. pseudomallei* were sensitive to 4 mg/l of gentamicin, while other workers (Eickhoff *et al.*, 1970; Franklin, 1971) have reported the organism to be resistant to gentamicin. All 91 strains of *Ps. pseudomallei* tested in this series were resistant to gentamicin by the disk-diffusion technique, and on subsequent testing they were found to be resistant to levels greater than 8 mg/l of gentamicin by the agar-dilution method (Ashdown, 1979).

API 20E was originally designed for the identification of fermentative organisms of the Enterobacteriaceae, and in recent years the manufacturer has indicated that the system is suitable for the differentiation of non-fermentative Gram-negative bacilli. Morris *et al.* (1978) have described the problems associated with API 20E for the identification of saccharolytic pseudomonads but they found that the system can be adequately used to separate *Pseudomonas* as a genus from other genera. The advantages of the multitest strip such as space saving, speed in inoculation and reading of reactions, accuracy, simplicity, computerised index, and low cost over conventional tests tend to promote the use of the system in the clinical laboratory. In this study it was found that because of the distinctive profile numbers for *Ps. pseudomallei* and good correlation with conventional tests, API 20E can satisfactorily be used routinely in the diagnostic laboratory as a basis for screening and presumptive identification of *Ps. pseudomallei*.

By complementing API with tests for heat stability of alkaline phosphatase, resistance to colistin and gentamicin, oxidative attack only of glucose and acid from maltose, a simple feasible system for positive identification of *Ps. pseudomallei* without the need of specific antiserum is available to the small clinical laboratory.

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