DIFFERENTIATION OF NONFERMENTATIVE GRAM-NEGATIVE BACILLI IN THE CLINICAL LABORATORY

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INTRODUCTION

Many nonfastidious, nonfermentative Gram-negative bacilli have become important in medical bacteriology in recent years. They have been recognised increasingly often as causes of clinical sepsis including septicaemia, meningitis, wound infections, pneumonia, and urinary tract infections (Gardner *et al.*, 1970; Pedersen *et al.*, 1970; Gilardi, 1972). *Pseudomonas aeruginosa*, the most commonly isolated species, is usually identified by its colonial morphology and pigment production (Gardner *et al.*, 1970). The other species of nonfermentative bacilli are more difficult to identify and special methods are required. Biochemical tests, such as peptone water sugars, commonly used to identify fermentative bacteria, like *Salmonella* and *Proteus* species, are not useful for the nonfermenters as most tests are scored as negative (Pickett and Pedersen, 1970b). Laboratory workers may not be aware of the characteristics of these nonfermenters with the result that many species may be wrongly identified or dismissed as contaminants.

This study describes a rapid and simplified system for the differentiation and identification of nonfermentative bacilli, other than the pyocyanogenic strains of *Ps. aeruginosa*, encountered frequently in clinical specimens. This system was drawn up after examining 332 strains of these organisms. The major characteristics of certain species are also described herein.

MATERIALS AND METHODS

The organisms examined in this study were isolated from routine clinical specimens collected at the University Hospital, Kuala Lumpur, between September 1971 and January 1973. The clinical specimens were mainly respiratory tract secretions (sputum, tracheal aspirates and throat swabs), urine, blood and wound swabs. Other specimens included CSF, ear discharge, eye swabs and high vaginal swabs. The specimens were cultured routinely on blood agar and MacConkey agar plates. In addition, chocolate agar plates were used for all specimens except urine and wound swabs. The cultures were incubated at 37°C for 1 to 2 days. Nonfermentative bacilli were recognised by their failure to produce acid in 1% glucose peptone water or in Kligler iron agar (Difco). In the Kligler medium, an alkaline or neutral reaction was produced in the slant and butt portions of the tube after overnight incubation. Motility was determined by microscopic examination of a hanging drop from an 18 hour broth culture. Oxidase test was done by Kovacs' method, using young cultures on nutrient agar plates. The following tests and media were also used:-

1) Oxidation of 1% glucose in Hugh and Leifson OF medium.
2) Oxidation of 10% lactose agar slope.
3) Reduction of nitrate to nitrite (Cook, 1950).
4) Deoxyribonuclease activity in DNase Test agar (Difco).
5) Aesculin hydrolysis in aesculin broth medium.
6) Fluorescein production in Seller's differential agar (Difco).
7) Arginine dihydrolase test in arginine agar.

8) Lysine and ornithine decarboxylase activities in Moeller's medium.
9) Gelatinase activity in nutrient gelatin medium.
10) Indole production in tryptone water and tested with Ehrlich's reagent.
11) Growth at 42°C in nutrient broth.
12) β-galactosidase activity in ONPG broth.

The media and methods used were as described by Cowan and Steel (1974), except the tests for nitrate reduction, deoxyribonuclease activity and fluorescein production. All these tests were selected from the identification schemes of Pickett and Pedersen (1970a, b) and Gilardi (1971a).

The strains were tested against chemotherapeutic agents by the disc-agar diffusion method (Bauer *et al.*, 1966) using Oxoid Diagnostic sensitivity test agar.

RESULTS

Table 1 presents the biochemical reactions most useful for differentiating non-motile nonfermentative Gram-negative bacilli. *Acinetobacter* species were oxidase negative while *Moraxella* and *Flavobacterium* species were oxidase positive.

A. anitratus (Herellea vaginicola, Achromobacter anitratum, Acinetobacter calco-aceticus) was identified by its strong oxidation with acid production in 1% glucose OF medium and 10% lactose agar. *A. lowffi (Mima polymorpha)* failed to oxidise glucose. Both species were nitrate negative. Microscopically, they appeared as short fat rods resembling cocci.

The nonsaccharolytic *Moraxella* species were identified readily by their susceptibility to penicillin and diplobacillary appearance in the Gram stain.

The flavobacteria gave positive reactions for glucose oxidation, deoxyribonuclease activity and aesculin hydrolysis. Colonies were usually pale yellow in the case of *F. meningosepticum* and bright yellow in the other spe-

Table 1

Differential reactions of non-motile nonfermentative Gram negative bacilli*.

Organism	Oxidase	Oxidation		DNase	Aesculin	Additional tests
		1% glucose	10% lactose			
Acinetobacter anitratus	–	+	+	–	–	Mainly coccoid forms
Acinetobacter lowffi	–	–	–	–	–	Nitrate negative
Moraxella	+	–	–	–	–	Diplobacilli. Penicillin sensitive.
F. meningosepticum	+	+	–	+	+	Light yellow. Indole + Typable.
Flavobacterium (other)	+	+	–	+/–	+	Usually bright yellow

* + = positive, – = negative, (+) = delayed positive > 2 days,
+/– = many positive, some negative, –/+ = many negative, some positive.

Table 2

Differential reactions of motile nonfermentative Gram-negative bacilli*.

Organism	Oxidase	Oxidation		Arginine dihydrolase	Fluorescein production	DNase	Aesculin	Additional tests
		1% glucose	10% lactose					
Ps. aeruginosa	+	+	−/+	+	+	−/+	−	Growth at 42°C
Ps. fluorescens	+	+	−/+	+	+	−	−	Gelatin + | No growth
Ps. putida	+	+	−/+	+	+	−	−	Gelatin − | at 42°C
Ps. pseudomallei	+	+	+	+	−	−	+/−	Wrinkled colonies
Ps. cepacia	+	+	+	−	−	−	+	Lysine +. ONPG +
Ps. maltophilia	−	(+)	−	−	−	+	+	Lysine +. Brown
Ps. putrefaciens	+	−/+	−	−	−	+	−	H_2S +. Brown/Orange
Other *Pseudomonas* species	+	+/−	−	−	−	−/+	−	Polar flagella
Alcaligenes species	+	−	−	−	−	−	−	Peritrichous flagella

* + = positive, − = negative, (+) = delayed positive > 2 days, +/− = many positive, some negative, −/+ = many negative, some positive.

cies. Indole production was usually weak or delayed. *F. meningosepticum* could also be identified by positive slide agglutination tests with specific antiserum (Difco). These bacteria had unusual antibiotic susceptibility patterns. They were all resistant to polymixin B but sensitive to rifampicin and novobiocin. Many strains were also sensitive to erythromycin.

Table 2 presents the reactions useful for differentiating motile nonfermentative bacteria, mainly *Pseudomonas* species. These organisms were oxidase positive with one exception, *Ps. maltophilia.* Many species of *Pseudomonas* oxidise glucose; some even attack 10% lactose.

The three species *Ps. aeruginosa, Ps. fluorescens* and *Ps. putida* gave positive reactions for arginine dihydrolase activity and fluorescein production. *Ps. aeruginosa* grew at 42°C but not the other two species. *Ps. fluorescens* could be differentiated from *Ps. putida* by its gelatinase activity. All strains of these fluorescent pseudomonads were susceptible to polymixin B.

Ps. cepacia (Ps. multivorans, Ps. kingii) was detected by its ability to oxidise 10% lactose and hydrolyse aesculin. This species gave negative reactions for arginine dihydrolase and deoxyribonuclease tests, and positive reactions for lysine decarboxylase and ONPG tests. *Ps. cepacia* isolates were resistant to polymixin B but susceptible to the sulphonamides and chloramphenicol.

Ps. maltophilia was the only motile species that was oxidase negative. It was recognised by its deoxyribonuclease activity and hydrolysis of aesculin. Most strains oxidised glucose slowly while maltose was oxidised more readily. This species also had lysine decarboxylase activity. In addition, *Ps. maltophilia* was proteolytic on blood agar and colonies often had a brown or dirty yellow pigment.

Ps. putrefaciens was identified readily by its production of abundant hydrogen sulphide, resulting in blackening of the butt of Kligler iron agar. Glucose was usually not oxidised. Colonies on nutrient agar had a brown to orange pigment. This species also had deoxyribonuclease and ornithine decarboxylase activities.

The remaining groups of nonfermentative bacilli required further tests for species identification. Flagellar staining must be used to differentiate the nonglucolytic *Pseudomonas* species from *Alcaligenes* species.

Table 3

Clinical isolates of nonfermentative Gram-negative bacilli.

Organism	No. of strains
A. anitratus	142
A. lwoffi	19
Moraxella species	11
F. meningosepticum	15
Flavobacterium (other)	24
Ps. aeruginosa (apyocyanogenic)	9
Ps. fluorescens	8
Ps. putida	6
Ps. cepacia	33
Ps. maltophilia	19
Ps. putrefaciens	2
Ps. species (glucolytic)	4
Alkaline organisms	23
Pseudomonas species (non-glucolytic) *Alcaligenes* species	
Miscellaneous	17
Total	332

Table 3 presents the clinical isolates of nonfermentative bacilli examined in this study. Using this system of identification, over 80% of isolates were identified to genus and species. The most commonly encountered species was *A. anitratus* with 142 isolates (42.8%).

Twenty three strains (6.9%) were called alkaline organisms as flagella staining had not been done to differentiate the nonsaccharolytic *Pseudomonas* species from *Alcaligenes* species. Seventeen strains (5%) could not be assigned to any recognised group or genus. *Ps. pseudomallei* was not found among the isolates in this study. During the 17 months period of study, ending January 1973, nonfermentative Gram-negative bacilli, other than *Ps. aeruginosa*, accounted for 8.9% of all Gram-negative rods isolated from clinical specimens in this hospital.

DISCUSSION

Von Graevenitz (1973) recommended that each laboratory should choose a system of identification of nonfermentative Gram-negative bacteria most suitable for its needs. He also stated that an identification system was useful only if it employed a minimum number of tests to identify the maximum number of species. The identification system presented here is simple and practical for routine use. It uses between 4 and 10 tests (usually 5 or 6 tests) to identify the majority of clinical isolates. Most species can be identified within 2-3 days after their isolation from clinical specimens. In addition, the media used are easy to prepare and the biochemical tests are not difficult to perform or interpret. Motility, oxidase reaction and ability to oxidise glucose are the important primary features in the identification of these bacteria and they must be determined accurately.

Although none of the isolates examined was identified as *Ps. pseudomallei*, the aetiological agent of meliodosis, this species can be identified readily using this system (Table 2). This organism gives positive results for all tests except fluorescein production and deoxyribonuclease activity (Pickett and Pedersen, 1970b; Gilardi, 1971a). A characteristic feature of this pseudomonad is the wrinkled appearance of colonies after 3 days incubation at 37°C (Von Graevenitz, 1973; Jayanetra *et al.*, 1975). *Ps. pseudomallei* can also be differentiated from the fluorescent pseudomonads by its resistance to polymixin B (Gilardi, 1971b).

Antibacterial susceptibility testing may be useful as aids in the identification of certain nonfermenters (Pedersen *et al.*, 1970; Gilardi, 1971b). *Moraxella* species are detected readily by their susceptibility to penicillin. Resistance to polymixin B appears to be characteristic of the *Flavobacterium* species and two pseudomonads, *Ps. cepacia* and *Ps. pseudomallei*. The flavobacteria can also be recognised by their susceptibility to rifampicin and novobiocin.

SUMMARY

A rapid and simplified system for the differentiation of nonfermentative Gram-negative bacilli, encountered frequently in clinical specimens, is presented for use in the clinical laboratory. Nonfermentative bacteria can be grouped initially by the motility, oxidase and OF glucose reactions. This grouping simplifies the choice of additional tests for further identification. The additional tests included Gram stain, acid production from 10% lactose agar, nitrate reduction, arginine dihydrolase activity, fluorescein production, deoxyribonuclease activity, hydrolysis of aesculin, growth at 42°C, gelatinase activity and susceptibility to antibiotics.

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