

## Methylene blue reduction disc test for the identification of *Pseudomonas pseudomallei*

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### Summary

A methylene blue reduction disc test to detect carbohydrate utilization has been adapted for the identification of *Pseudomonas pseudomallei*. The test is easy to perform, is not time consuming, can be read without difficulty and gives reliable results.

### 1. INTRODUCTION

Melioidosis has been diagnosed in approximately 30% of the porcine abscesses submitted at slaughter in northern Queensland (Thomas 1981). The causative organism, *Pseudomonas pseudomallei*, has also been isolated from various other animal species and from the soil in the Townsville area (Laws and Hall 1963, 1964; Thomas, Forbes-Faulkner and Parker 1979; Thomas 1981). Identification, however, can often be difficult if the organism is not a regular isolate in the laboratory.

Acid production from a variety of carbohydrate media is the routine method for identifying isolates in the clinical laboratory. Variability in the results, however, has always been a problem with the pseudomonads, including *Ps. pseudomallei* (Sears and Gourlay 1928; de Lajudie and Brygoo 1953; Wetmore and Gochenour 1956; Biegeleisen, Mosquerra and Cherry 1964; Laws 1964; Zierdt and Marsh 1971).

There have been two methods that have given satisfactory results in the pseudomonads (Gilardi 1968, 1971; Schubert and Esanu 1977). Both of these methods relied on carbohydrate utilization tests either by growth of the organism on agar slopes (Gilardi 1968, 1971) or growth of the organism with accompanying reduction of methylene blue in the broth medium as a positive result (Schubert and Esanu 1977). Because of the simplicity of the latter test, which did not include *Ps. pseudomallei*, it was used in the present study to examine 50 strains of *Ps. pseudomallei* isolated at this laboratory in an attempt to obtain a satisfactory identification test for routine laboratory use.

### 2. MATERIALS AND METHODS

#### Strains

Fifty strains of *Ps. pseudomallei* were isolated from soil (21), water (3), pigs (13), sheep (6), goats (3), birds (2), a horse (1) and a tree kangaroo (1). They were identified according to the method of Laws (1964) and were all positive by slide agglutination to a known positive rabbit antiserum to *Ps. pseudomallei*.

### Media

The media used were those described by Schubert and Esanu (1977). They consisted of a standard mineral salt base medium either with methylene blue (SMBM) or without methylene blue (SMB). The SMB was distributed in 7 mL bijou bottles (3 mL amounts) and the SMBM in either 7 mL bijou bottles (3 mL amounts) or 100 × 13 mm Corning cell culture tubes with screw capped lids (4 mL amounts). After dispensing, the media were autoclaved at 121°C for 20 min.

### Disc preparation

The method of Schubert and Esanu (1977) was followed with minor changes. An office punch was used to prepare 6 mm Whatman filter paper No. 1 discs instead of using bought sterile discs. The dried discs were stored in small vials at 4°C until required. A layer of silica gel was placed under a foam circle at the bottom of the vial to control moisture content.

A set of control discs using sterile distilled water in place of the test substrate was also prepared.

### Test substrates

Sole carbon sources used were acetamide, arabinose, arginine, citrate, dulcitol, galactose, glucose, inositol, lactose, lysine, mannitol, ornithine, polyethylene glycol, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose and xylose.

### Methods

The basic test was performed by inoculating a bottle of SMB with a colony from a 24 h blood agar culture of the test strain. After shaking gently, 0.1 mL was transferred into a set of 21 bottles or tubes of SMBM (20 for carbon source addition and 1 for control purposes). Sterile forceps were used to transfer the control and test discs, one per bottle or tube.

**Test 1.** Duplicate sets in both bottles and tubes were processed for the 50 strains of *Ps. pseudomallei*. These were incubated at 37°C and read daily for 7 days and then weekly to the 28th day.

**Test 2.** Duplicate sets of bottles were processed for the 50 strains of *Ps. pseudomallei*. One set was incubated at 28°C and the other at 37°C.

**Test 3.** Fifteen strains of *Ps. pseudomallei* were used for the last test. These were set up in duplicate for 5 test discs (arginine, glucose, inositol, lysine and ornithine plus control) using bottles only. One set of bottles was overlaid with sterile liquid paraffin oil to a depth of approximately 3 mm. All bottles were incubated at 37°C.

### Reading of results

A negative test was rated when the liquid retained the blue colour of the control tube and no growth was visible. A positive test was scored when there was reduction of the methylene blue with growth due to the utilization of the test substrate.

## 3. RESULTS

**Test 1.** The 50 strains of *Ps. pseudomallei* were consistently positive for the substrates arginine, citrate, dulcitol, galactose, glucose, inositol, lysine, mannitol, ornithine, sorbitol and trehalose. The results were obtained within 2 days except for lysine and ornithine, where a positive result was achieved within 2 to 7 days. The strains were consistently negative for acetamide, arabinose, lactose, polyethylene glycol, raffinose, rhamnose and xylose. Salicin and sucrose gave variable results (9 strains giving a positive result with salicin and 2 strains with

sucrose) but, when they were positive, the results were not noted until 21 to 28 days. The results for these two substrates were not consistent within the strains. Use of bottles or tubes for the test made no difference to the test results.

**Test 2.** No variation of the test results occurred when using either 28°C or 37°C.

**Test 3.** The paraffin oil overlay made no difference to the test results, except that positive results were obtained faster in most cases when the overlay was used.

It was noted that, during the test, the disc takes up the methylene blue dye readily, thus becoming blue. With decolorization of the medium there was often a simultaneous loss of colour in the disc, which distinguished it from a negative disc where it always remained blue. This ability of the disc to decolorize appears to be a function of its preparation. The discs were less likely to decolorize if they were left in the drying oven at 96°C after the initial drying period.

#### 4. DISCUSSION

King and Phillips (1978) in their study of pseudomonads and related bacteria acknowledged the value of using substrate utilization tests for the identification of these genera. However, they also appreciated the difficulty in performing such tests, and used acid production from carbohydrates as their criterion for identification.

The use of acid production from media based on peptone and containing a variety of carbohydrates as a sole carbon source has always proved difficult with *Ps. pseudomallei*. This is because the organism can also break down the peptone in the medium, producing alkaline substances in quantities sufficient to neutralize the acids resulting from the breakdown of the carbohydrates (Zierdt and Marsh 1971). Sears and Gourlay (1928) found that any change in the amount of peptone in the medium would cause varying carbohydrate reactions, while Wetmore and Gochenour (1956) also obtained varying results with their medium when there was any change in its nitrogen content.

Substrate utilization test media contain no peptone and rely on the growth of the organism in the presence of certain carbohydrates. The use of a simple utilization test such as the methylene blue reduction disc test of Schubert and Esanu (1977) proved to be easy to perform, could be read without difficulty and gave reliable results. The method proved less time consuming because there was need to dispense only one set of test basal media. Once a series of test substrate discs has been prepared, they will remain stable at 4°C for at least a year (unpublished data) and can thus be used as required. This is of value if *Ps. pseudomallei* is not a common isolate in the laboratory.

The reading of the test depends on the reduction of methylene blue that occurs on utilization of the substrate. *Pseudomonas* species, including *Ps. pseudomallei*, are known to reduce this dye and are therefore suitable for this test. The decolorization occurs throughout the medium except at the surface where oxygen diffusion causes the top layer to remain blue. It is important that the tubes or bottles are not shaken before reading as introduced oxygen will obscure the clearing zone seen where reduction has occurred. The tube, being deeper than the bottle, allows for easier reading of the reduction zone. However, bottles are easier to handle and store when many tests are being done.

The best method of stopping the re-oxidation of the methylene blue is to overlay the media with sterile paraffin oil. Once the positive result is obtained the whole medium is clear and, without introduced oxygen, will remain so even if shaken. However, although it allows for easier reading of the test, it is more time consuming and makes cleaning more difficult. The results obtained in this trial showed that similar results were recorded both with and without the overlay. The other reason for using the overlay technique is to reduce the chance of false

positive readings, especially where a late reaction is obtained, for example with lysine and ornithine. However, as similar results were obtained with both methods, the overlay technique proved to be unnecessary.

The original method recommended incubation of pseudomonads at 28°C. We found no difference in the results if incubated at 37°C, and chose the latter because of ease in laboratory handling, as well as the fact that most of the isolates obtained in the laboratory are recovered from animal hosts as a pathogen. We also followed the reactions for 28 days whereas the original method called for 2 days. The results have shown that 2 days would be sufficient for the identification of *Ps. pseudomallei*. For this period of time, sucrose and salicin would give negative results as would also lysine and ornithine in most cases.

There was no difference in the results obtained from animal or soil strains, which agrees with antibiotic sensitivity testing data collected in this laboratory (Thomas, Forbes-Faulkner and Duffield 1981). The results obtained agree with those of workers who have used similar substrate utilization tests to differentiate *Ps. pseudomallei* from other pseudomonads (Gilardi 1968, 1971), or to identify the melioidosis organism (Dodin and Galimand 1976).

Our results indicate that the use of the methylene blue reduction disc test in the laboratory should be of great assistance in the identification of *Ps. pseudomallei*.

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