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Evaluation of Culture Techniques for Isolation of *Pseudomonas pseudomallei* from Soil

LESLIE R. ASHDOWN* AND SUE G. CLARKE

Anton Breinl Centre for Tropical Health and Medicine, James Cook University of North Queensland, Townsville, Queensland 4811, Australia

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Three selective enrichment broths and four selective agar media were evaluated for their ability to support the growth of *Pseudomonas pseudomallei* both at 35°C and at ambient temperature (range, 20 to 32°C; mean, 25°C). Colony counts of 50 strains of *P. pseudomallei* and recovery studies with 1 soil strain in 60 simulated soil samples demonstrated that enrichment with Trypticase soy broth incorporating 5 mg of crystal violet per liter and 20 mg of colistin per liter (CVCB) and subculture to Ashdown medium supported the growth of all 50 strains and produced the highest recovery rates with the greatest suppression of other soil flora. An enrichment broth of MacConkey broth (purple) incorporating 10 mg of crystal violet per liter, 5 mg of bromcresol purple per liter, 25 mg of gentamicin per liter, and 650 mg of streptomycin per liter showed greater suppression of soil bacteria than CVCB, but it failed to support the growth of three strains of *P. pseudomallei*. Recovery rates were essentially the same irrespective of whether the soil samples were incubated at 35°C or at ambient temperature, provided cultures were incubated in protected shade for an extended period. This is an important feature for field work in large-scale epidemiological surveys in which resources are limited.

Pseudomonas pseudomallei is a soil saprophyte of certain tropical regions, presumably occupies an ecological niche similar to that occupied by *P. cepacia*, and causes melioidosis, a disease of animals and humans. In humans, the disease varies greatly in its clinical presentation. It may range from an asymptomatic carrier state manifested only by the presence of specific circulating antibodies to an overwhelming pneumonia or septicemia which, if not appropriately treated, has an 80 to 90% mortality rate, with death frequently occurring 1 to 2 days after the onset of symptoms. Disease transmission is predominantly by overt or covert wound exposure to contaminated soil or surface water but also occurs by inhalation and perhaps by ingestion (6, 9, 10).

Studies to determine the distribution and ecology of *P. pseudomallei* in the environment rely on recovering the bacterium from soil or water samples or both. A problem in ascertaining the presence of *P. pseudomallei* in these types of specimens is isolating and differentiating the bacterium from the heterogeneous population of organisms that may be present in the environment (11). A variety of methods to retrieve *P. pseudomallei* selectively from soil have been devised (1, 7, 8, 12, 13, 15), but to date no comparative studies to determine the efficacies of selective isolation media have been documented.

The objectives of this investigation were to compare three selective enrichment broths and four selective isolation agar media for their abilities to sustain growth of *P. pseudomallei* and to permit easy recognition and maximum recovery of *P. pseudomallei* in simulated positive soil, by using inexpensive methods that would be applicable to field work in large-scale epidemiological surveys in which resources are limited.

MATERIALS AND METHODS

Test strains. Fifty isolates (32 from humans, 6 from animals, and 12 from soil) of *P. pseudomallei* were used in these studies. All strains were identified as *P. pseudomallei* by colonial morphology, biochemical reactions, and agglutination with specific antiserum (2). Strains were propagated on nutrient agar slants and stored at 4°C. With sterile physiological saline, working suspensions were prepared from the 50 strains by comparing and adjusting visually the cell density with a 0.5 McFarland standard, and further dilutions were made from the standardized suspensions by the procedures described below.

Selective isolation media. The media evaluated were the selective enrichment broths described by Ashdown (CVCB) (1), Dodin and Galimand (T-BSS) (7), and Thomas and Forbes-Faulkner (MacB-Mod) (14) and the selective isolation agar media described by Ashdown (Ashdown medium) (3), Dodin and Galimand (T-BSA) (7), Ellison et al. (GCVA) (8), and McCormick et al. (Mac-VCN) (12). The selective supplement used for Mac-VCN was VCN Supplement (SR 101; Oxoid, Basingstoke, United Kingdom), while the selective agents for the other media were prepared in the laboratory by using assayed antibiotic or dye powders. The concentration of agar used in T-BSA was 15 rather than 30 g/liter, and the concentration of gentamicin used in Ashdown medium was 8 rather than 4 mg/liter as in the original descriptions (3, 7). Double-strength selective enrichment broth, aseptically dispensed in 25-ml aliquots in 100-ml containers, was used for the soil studies. Before use, selective agar media were dried for 1 h at 35°C to inhibit swarming of organisms and were inoculated in a manner designed to separate single colonies. A summary of the characteristics of the various media used is given in Table 1.

Evaluation of isolation media. The media were tested for their ability to support the growth of *P. pseudomallei* in pure culture. Five 10-fold dilutions were made in sterile physiological saline from the standardized suspensions of 50 strains of *P. pseudomallei*. Two sets consisting of each test medium and a nutrient broth or agar plate as a control were inoculated in duplicate with 50 μl from each of the last three dilutions. One set of inoculated culture media was incubated at 35°C for 48 h, while the other set was incubated in the shade at ambient temperature (range, 20 to 32°C; mean,

* Corresponding author.

TABLE 1. Isolation media for *Pseudomonas pseudomallei*

Medium	Base	Differential agent(s)	Selective agent(s)	Reference
CVCB	Trypticase soy broth	None	Crystal violet (5 mg/liter), colistin (20 mg/liter)	1
T-BSS	Buffered salt solution with L-threonine (1.2 g/liter)	None	None	7
MacB-Mod	MacConkey broth (purple)	None	Crystal violet (10 mg/liter), bromcresol purple (5 mg/liter), gentamicin (25 mg/liter), streptomycin (650 mg/liter)	14
Ashdown	Trypticase soy agar with glycerol (4%)	Neutral red, crystal violet	Crystal violet (5 mg/liter), gentamicin (8 mg/liter)	3
T-BSA	Buffered salt solution with L-threonine (1.2 g/liter)	None	None	7
GCVA	Nutrient agar with glycerol (3%)	Crystal violet	Crystal violet (5 mg/liter)	8
Mac-VCN	MacConkey agar	Neutral red	Vancomycin (3 mg/liter), colistin (7.5 mg/liter), nystatin (12,500 U/liter)	12

25°C) for 96 h. Growth in isolation medium was recorded for each strain as no growth to 3+ growth when compared with growth in the control medium (3+).

Simulated positive soil samples. To test the recovery of *P. pseudomallei* among mixed soil flora, we used 20 random samples obtained from each of three soil varieties, solodic, red earth, and alluvial (in the United States, natrustalf, paleustalf, and haplaquept, respectively), which are the common soil types in the environs of Townsville, Queensland, Australia, and which had been found previously to be negative for *P. pseudomallei*. From each sample, 50-g aliquots were transferred to clean, disposable containers and were seeded with 10^3 CFU of a soil strain of *P. pseudomallei* (3) capable of growing in all isolation media. Two sets of samples were used. Each sample was emulsified with 100 ml of sterile distilled water to obtain a homogeneous soil suspension and was left overnight at ambient temperature to allow the soil to sediment. Equal volumes (25 ml) of supernatant from each sample were added to each of the three double-strength selective enrichment broths, and one set of samples was incubated without shaking for 48 h at 35°C while the other set was incubated without shaking in the shade at ambient temperature (range, 20 to 32°C; mean, 25°C) for 96 h. The surface layer of each undisturbed enrichment broth was sampled with sterile swabs and then inoculated onto each selective agar medium. The inoculated plates from the set of samples which had been incubated at 35°C were incubated at 35°C for 48 h, while the plates from other set were incubated in the shade at ambient temperature (range, 20 to 32°C; mean, 25°C) for 72 h. Nutrient agar plates inoculated with each enrichment broth seeded with *P. pseudomallei* were used as controls for recovery of *P. pseudomallei*. A growth control was also made by using phosphate-buffered saline instead of enrichment broth; this control was processed in the same way as each set and inoculated on nutrient agar. Quantitation of soil flora was made by comparing the amounts of growth on the selective media with that on the control medium. Results were based on observation of a 75% reduction in the soil flora compared with that on the control medium.

Each colonial type isolated on the four selective agar media was subcultured onto nutrient agar, incubated at 35°C for 24 h, and tested for cytochrome oxidase. Isolates which were positive for oxidase activity were then examined for reactivity with antiserum against *P. pseudomallei* by slide agglutination. Isolates which agglutinated with specific antiserum were confirmed as *P. pseudomallei* according to the criteria of Ashdown (5).

Statistical analysis. Analysis of data was carried out by Wilcoxon's rank sum test.

RESULTS

Laboratory evaluation of media. The ability of each selective medium to support the growth of *P. pseudomallei* both at 35°C and at ambient temperature is given in Table 2. Growth was supported by all 50 strains at an inoculum of 100 CFU by the control (nutrient agar) and by CVCB, Ashdown medium, GCVA, and Mac-VCN both at 35°C and at ambient temperature. Three strains failed to grow in MacB-Mod, and growth of two strains was not supported by T-BSS and T-BSA with either of the two incubation period-and-temperature formats. Experiments which compared the growth rates of 50 strains of *P. pseudomallei* in CVCB and T-BSS and in CVCB and MACB-Mod were performed. Growth in CVCB was superior or equal to growth in T-BSS at both

TABLE 2. Recovery of 50 strains of *P. pseudomallei* from seven selective media

Medium	No. of strains recovered[a] at:	
	35°C[b]	Ambient temp[c]
Nutrient agar	50	50
CVCB	50	50
T-BSS	48	48
MacB-Mod	47	47
Ashdown	50	50
T-BSA	48	48
GCVA	50	50
Mac-VCN	50	50

[a] Inoculum size, 10^2 CFU.
[b] Incubation period, 48 h.
[c] Range, 20 to 32°C; mean, 25°C; incubation period, 96 h.

TABLE 3. Comparison of growth of 50 strains of *P. pseudomallei* in three selective enrichment broths

Growth conditions and results[a]	No. of isolates	
	35°C[b]	Ambient temp[c]
CVCB vs T-BSS		
Superior in CVCB	36	36
Superior in T-BSS	0	0
Similar	14	14
CVCB vs MacB-Mod		
Superior in CVCB	6	6
Superior in MacB-Mod	0	0
Similar	44	44

[a] Inoculum size, 10^2 CFU.
[b] Incubation period, 48 h.
[c] Range, 20 to 32°C; mean, 25°C; incubation period, 96 h.

35°C and ambient temperature (Table 3), with two strains failing to grow in T-BSS. A comparison of growth at both temperatures in MacB-Mod and CVCB showed that 44 strains (88%) grew equally well in the two media. The growth of the other six strains was superior in CVCB, with three strains failing to grow in MacB-Mod. The comparison of growth on each of the four selective agar media and nutrient agar controls for inoculum sizes of 25, 50, and 100 CFU is summarized in Table 4. Amounts of growth on Ashdown medium, GCVA, and Mac-VCN were comparable and were slightly less than those obtained on the controls for all dilutions. However, there was significantly less growth on T-BSA than in the control medium at both temperatures of incubation.

Simulated positive soil samples. In contrast to the results based on pure cultures, the recovery of *P. pseudomallei* from simulated soil samples was best achieved with either CVCB or MacB-Mod as an enrichment, while T-BSS was the least efficacious (Table 5). The selective agar medium giving the best results was Ashdown medium, followed by Mac-VCN, while GCVA and T-BSA were the least efficacious for *P. pseudomallei* recovery. However, there was no statistically significant difference between Ashdown medium and Mac-VCN by Wilcoxon's rank sum test at $P < 0.05$. By using the combination of either CVCB or MacB-Mod enrichment and Ashdown selective medium, *P. pseudomallei* was recovered from almost all soil samples. There was no significant difference in isolation rates between incubation at 35°C for 48 h and at ambient temperature for 96 h, and the soil type did not appear to influence isolation rates. Soil floras grew in all five streaked sections of the control medium from all 60 samples of soil. The abilities of each of the three selective enrichment broths combined with each of the four selective agar media to suppress soil flora are given in Table 6. The greatest suppression of the soil flora occurred with MacB-Mod and Ashdown medium, followed by CVCB and Ashdown medium. There was less inhibition of soil flora demonstrated on Mac-VCN and GCVA, while T-BSS and T-BSA yielded significant numbers of soil bacteria. In two soil samples, despite prior plate drying, swarming *Proteus* cells overgrew GCVA and T-BSA but not Ashdown medium or Mac-VCN after enrichment with CVCB and T-BSA. Swarming did not occur with MacB-Mod. The soil flora was suppressed slightly more at 35°C than at ambient temperature, but the difference did not appear to be of practical importance.

At an inoculum size of 10^3 CFU/50 g of soil, *P. pseudomallei* was recovered from 95% or more of simulated soil specimens with either CVCB or MacB-Mod and Ashdown medium (Table 5). Nonetheless, most *P. pseudomallei* isolates were not the predominant soil bacteria, and recovery was made by carefully examining all colonial varieties on the selective agar medium. The purple, rugose colonies of *P. pseudomallei* on Ashdown medium after 2 to 3 days of incubation made recognition easier. Some bacteria, *P. cepacia* in particular, initially formed pale purplish colonies which could be easily confused with *P. pseudomallei* by the uninitiated. However, within 1 to 2 days, these colonies became brown and distinct from *P. pseudomallei*.

DISCUSSION

The most important features of a selective isolation medium are the ability to support the growth of the target organism and the ability to inhibit floras that may overgrow the organism. In this study, CVCB and MacB-Mod were the most effective enrichment broths, as shown in Tables 5 and 6. CVCB was beneficial because it supported the growth of all 50 *P. pseudomallei* strains, but it had the disadvantage of allowing the multiplication of *Proteus* spp. and other colistin-resistant gram-negative bacteria. Although this did not result in reduced isolation rates on Ashdown medium, the incorporation of gentamicin at a level of 8 mg/liter would probably improve the efficacy of the CVCB enrichment. MacB-Mod was more selective than CVCB, but it was not capable of supporting the growth of three (6%) of the test strains, presumably because of the high (25 mg/liter) level of gentamicin incorporated in the broth. Strains of *P. pseudomallei* susceptible to levels of greater than 15 mg/liter are not uncommon (4), and the use of MacB-Mod may lead to diminished isolation rates despite the greater ability of this broth to suppress soil floras.

Organisms frequently encountered in soil types of Townsville's environs include *P. aeruginosa*, *P. alcaligenes*, *P. cepacia*, *P. fluorescens*, and *P. gladioli*; *Comamonas*, *Flavobacterium*, and *Bacillus* spp.; fungi; and others. Many of these bacteria are inherently resistant to both gentamicin and colistin, antibiotics which are commonly used as selective agents to suppress soil bacteria during culture. It was thought that soil isolation media based on inorganic salts and threonine (7) would overcome this problem. However, the results obtained in this study with basal salts enrichment broth (T-BSS) and selective agar (T-BSA) were disappointing, and these were the least sensitive of all media tested for

TABLE 4. Evaluation of four selective agar media with 150 quantitative cultures of 50 strains of *P. pseudomallei*

Quantitative culture and no. of CFU	% Growth on selective medium relative to controls			
	Ashdown medium	T-BSA	GCVA	Mac-VCN
35°C[a]				
100	94	59	97	92
50	93	50	95	89
25	93	41	95	91
Ambient temp[b]				
100	95	57	96	93
50	93	51	94	90
25	92	44	94	91

[a] Incubation period, 48 h.
[b] Range, 20 to 32°C; mean, 25°C; incubation period, 96 h.

TABLE 5. Recovery of *P. pseudomallei* from 60 seeded solodic, red earth, and alluvial soil samples with various enrichment broths and selective isolation agar media

Temp and medium	% of cultures positive for *P. pseudomallei* in indicated soil[a]									P^b
	Solodic			Red earth			Alluvial			
	CVCB	T-BSS	MacB-Mod	CVCB	T-BSS	MacB-Mod	CVCB	T-BSS	MacB-Mod	
35°C[c]										
Ashdown	95	50	100	100	55	95	100	55	95	
T-BSA	50	40	55	55	45	50	55	40	55	<0.0002
GCVA	55	45	50	55	40	50	50	40	55	<0.0002
Mac-VCN	80	55	85	80	50	80	85	60	80	0.077
Ambient[d]										
Ashdown	100	55	100	100	50	95	95	55	100	
T-BSA	45	45	50	50	45	45	50	40	50	<0.0002
GCVA	45	50	55	50	45	55	50	40	55	<0.0002
Mac-VCN	85	55	80	75	45	80	85	40	75	0.077

[a] Inoculum, 10^3 CFU/50 g of soil; $n = 20$ for each soil type.
[b] P values for Ashdown medium versus other media; $P < 0.05$ set as level of statistical significance.
[c] Incubation period, 48 h.
[d] Range, 20 to 32°C; mean, 25°C; incubation period, 96 h.

recovering *P. pseudomallei* from soil. In the present study, Ashdown medium was the best-performing selective agar. Although isolation rates with Mac-VCN were not significantly different from those with Ashdown medium, colonies of *P. pseudomallei* on Ashdown medium were more easily recognizable than those on Mac-VCN because they had a distinctive purple, rugose appearance after 2 to 3 days of incubation. Ashdown medium has been previously documented as a useful differential, selective medium for the isolation of *P. pseudomallei* from clinical specimens, such as sputum, which may contain large amounts of the normal body flora (16).

For epidemiological soil surveillance culture studies of *P. pseudomallei*, it is important to devise methods that are inexpensive and that can be readily applied in the field with large numbers of samples by staff without necessarily high levels of technical skills. The results of this study demonstrate that when ambient temperatures are high (range, 20 to 32°C), as in the tropics, there is no significant difference between isolation rates of *P. pseudomallei* from soil after laboratory incubation (35°C) and after incubation in the field, provided that the incubation area is sheltered from direct sun, rain, and wind and the incubation times are prolonged.

In summary, from our experience with several commonly used media for isolation of *P. pseudomallei* from soil, optimal recovery requires the use of a selective enrichment broth and a differential selective medium. In the present study, CVCB enrichment followed by subculture to Ashdown medium gave the best results. This performance was due to the selective enrichment action of CVCB and the ability of Ashdown medium to support the growth of *P. pseudomallei*, to yield characteristic colonies, and not to rely on fermentable carbohydrates for colonial differentiation.

TABLE 6. Suppression of soil floras from 60 *P. pseudomallei*-seeded solodic, red earth, and alluvial soil samples with various enrichment broths and selective isolation agar media

Enrichment and medium	No (%) of plates with 75% reduction of soil flora compared with control after incubation at:	
	35°C[a]	Ambient temp[b]
CVCB		
Ashdown	50 (83)	49 (82)
T-BSA	33 (55)	30 (50)
GCVA	35 (58)	33 (55)
Mac-VCN	45 (75)	43 (72)
T-BSS		
Ashdown	23 (38)	22 (37)
T-BSA	11 (18)	13 (22)
GCVA	17 (28)	15 (25)
Mac-VCN	21 (35)	20 (33)
MacB-Mod		
Ashdown	51 (85)	52 (87)
T-BSA	36 (60)	35 (58)
GCVA	38 (63)	37 (62)
Mac-VCN	47 (78)	48 (80)

[a] Incubation period, 48 h.
[b] Range, 20 to 32°C; mean, 25°C; incubation period, 96 h.

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