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NOTES

Novel Selective Medium for Isolation of *Burkholderia pseudomallei*

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Isolation of *Burkholderia pseudomallei* currently relies on the use of Ashdown's selective agar (ASA). We designed a new selective agar (*Burkholderia pseudomallei* selective agar [BPSA]) to improve recovery of the more easily inhibited strains of *B. pseudomallei*. *B. pseudomallei*, *Burkholderia cepacia*, and *Pseudomonas aeruginosa* were used to determine the selectivity and sensitivity of BPSA. BPSA was more inhibitory to *P. aeruginosa* and *B. cepacia* and should make recognition of *Burkholderia* species easier due to distinctive colony morphology. BPSA also inhibited *Enterococcus*, *Escherichia*, *Staphylococcus*, and *Streptococcus*. These results indicate that BPSA is a potential replacement for ASA.

Laboratory diagnosis of acute septicemic melioidosis is usually made by conventional blood culture techniques. In these circumstances a rich nonselective agar is adequate (6). However, isolation of *Burkholderia pseudomallei* is more difficult in clinical specimens from nonsterile body sites. Isolation from sputum is particularly difficult when low numbers of *B. pseudomallei* are present and there is an abundant commensal upper respiratory tract flora. The isolation of other nonfermentative gram-negative species that morphologically resemble *B. pseudomallei* further compounds the difficulties experienced in the bacteriological diagnosis of melioidosis (5).

Ashdown's selective agar (ASA) (1) is the currently favored medium for the isolation and presumptive identification of *B. pseudomallei* in areas where melioidosis is endemic. *B. pseudomallei* produces highly wrinkled circular purple colonies on ASA by 48 h. A report of *B. pseudomallei* detection by reverse transcription-PCR despite an absence of growth on ASA (2) suggests that significant improvements could be made to the isolation technique currently in use. It has also been noted in local clinical laboratory use that ASA is inhibitory to some strains of *B. pseudomallei* (T. J. J. Inglis and R. J. Hunter, Abstr. 100th Gen. Meet. Am. Soc. Microbiol., abstr. C-273, p. 193, 2000). In that study, comparisons of ASA with a nonselective, enriched agar medium indicated that the recovery of the persistently mucoid variant could be improved.

There has been no significant improvement in the isolation of *B. pseudomallei* from clinical specimens from nonsterile sites since the original development of ASA. The aim of the present study was to develop a selective agar (*Burkholderia pseudomallei* selective agar [BPSA]) that did not inhibit the persistently mucoid colony form of *B. pseudomallei* while maintaining selective activity against other bacteria.

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Media development. Each of the ingredients of Ashdown's selective medium (Trypticase peptone, 4% glycerol, 5 mg of crystal violet per liter, 50 mg of neutral red per liter, and 4 mg of gentamicin per liter), Nile blue, and nine potential carbon sources were tested for inhibition of colony formation and colony size and wrinkling of colony surfaces by individual inclusion in a minimal medium. In preliminary studies, Nile blue proved to be an effective indicator (Inglis and Hunter, Abstr. 100th Gen. Meet. Am. Soc. Microbiol.).

Primary carbon source. *B. pseudomallei* strains NCTC 13177 and BCC11, representative of nonmucoid and mucoid colony forms, respectively, three strains of *Burkholderia cepacia* (NCTC 10743, NCTC 10661, and WACC1185), and one strain of *Pseudomonas aeruginosa* were used to determine the optimum carbon source for the growth of all colony forms of *B. pseudomallei* while being deleterious to potential competitors. Each carbon source (maltose, glycerol, D-(−)-β-hydroxybutyric acid, salicin, succinic acid, sodium salicylate, D(+)-glucosamine, i-erythritol, and isobutyric acid) (200 or 20 mg/liter) was added to a minimal medium (K_2HPO_4, 7 g/liter; KH_2PO_4, 2 g/liter; $(NH_4)_2SO_4$, 1 g/liter; $MgSO_4$, 0.1 mg/ml). The control medium for this experiment contained no carbon source. The inoculum was prepared as 10-ml overnight cultures and then centrifuged at 300 × g for 15 min, after which the pellet was resuspended in 3 ml of cold water for 10 min and centrifuged for a further 10 min. The wash step was repeated and the pellet was resuspended in minimal medium with no carbon source. A 10-μl inoculation of approximately 10^8 CFU of each strain per ml was put into 10 ml of prewarmed (35°C) medium. There were two replicates for each treatment, and 5-h colony counts and 24-h spectrophotometry readings (450 nm) were taken in duplicate to calculate growth in each medium.

The lower concentration of maltose provided the highest colony counts and largest colony sizes of all the carbon sources tested, while being slightly inhibitory to some *B. cepacia* strains.

Antibacterial supplements. To determine if crystal violet, neutral red, or Nile blue is inhibitory to *B. pseudomallei* growth, 5, 100, and 20 mg of each, respectively, per liter was added

separately to standard methods agar (SMA) containing 20 mg of gentamicin per liter. Inocula of NCTC 13177, NCTC 10276, and BCC11 were prepared as described for the carbon source experiment, and suspensions of approximately 10^4 organisms/ml of water were spiral plated (Don Whitley, Shipley, United Kingdom) onto three replicate plates for each treatment. Colony counts were performed at 24 h for each strain and at 48 h for the nonmucoid strains. Three plates for each strain were streaked for single colonies, and the largest colony on each plate was recorded at 24 and 48 h after incubation at 35°C.

Crystal violet reduced colony counts of NCTC 13177 100-fold and reduced colony sizes of NCTC 10276 by 75% and BCC11 by 50%. Neutral red and Nile blue had no effect on colony counts but slightly reduced the colony size of NCTC 13177.

Glycerol. SMA was prepared with 0 and 4% glycerol to test if the incorporation of glycerol into solid media had any effect on the persistently mucoid strain. Glycerol (0 and 4%) was also added to a less rich medium (K_2HPO_4, 1.7 g/liter; KH_2PO_4, 0.45 g/liter; $MgSO_4 \cdot 7H_2O$, 0.12 g/liter; $CaCl_2$, 0.01 g/liter; NaCl, 10 g/liter) solidified with 1% bacteriological agar (Oxoid Ltd., London, United Kingdom). The glycerol was added after autoclaving when the medium had cooled to 40°C. Inocula were grown overnight, washed, and diluted to approximately 10^3 organisms/ml before spiral plating onto the test medium. Colony counts were performed at 24 and 48 h after incubation.

The addition of glycerol to SMA had no significant effect on colony counts; however, it significantly reduced colony size. Colonies were slower to appear and were very small (<0.1 mm) on the minimal medium containing glycerol.

BPSA preparation. BPSA comprised 23.5 g of SMA (BBL, Cockeysville, Md.), 4 g of maltose (Sigma, St. Louis, Mo.), and 100 mg of neutral red (Sigma) in 1 liter of distilled water sterilized at 134°C for 10 min (or 121°C for 15 min). The agar was allowed to cool to 40 to 45°C, and then 20 mg of gentamicin (Sigma) per liter and 1 ml of 20-g/liter Nile blue (dissolved in 1% dimethyl sulfoxide) were added after filter sterilization via a 0.2-μm-pore-size membrane (Pall Corporation, Ann Arbor, Mich.). Ten milliliters of glycerol (equivalent to 1%) (BDH, Merck P/L, Kilsyth, Australia) was added and the medium was placed onto a heated magnetic stirrer at 40°C for 5 min before plates were poured (18 ml/plate). ASA plates were prepared by Excel Laboratory Products, Belmont, Western Australia, Australia, and included batch numbers 213164, 214750, 216753, and 220269.

Bacterial strains used to evaluate BPSA. The following strains were used to evaluate BPSA: *B. pseudomallei* reference strains NCTC 10276 and 13177 and 48 wild strains from clinical and environmental specimens, which included the persistently mucoid variant WACC11; 11 *B. cepacia* wild strains representative of each genomovar (T. Pitt, PHLS, Colindale, United Kingdom); 51 wild strains of *P. aeruginosa* and 1 wild strain each of *Pseudomonas fluorescens*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas putida*, and *Pseudomonas stutzeri*; and 1 isolate each of *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), and *Streptococcus pyogenes* (ATCC 19615).

All strains were taken from 15% glycerol in brain heart infusion broth stock kept at −70°C, plated onto blood agar for a maximum of 3 days, transferred to 10 ml of Trypticase soy broth for 18 h of incubation prior to use in the experiments, and incubated in air at 35°C.

TABLE 1. Ranking system for surface wrinkling of single colonies of *B. pseudomallei* grown on solid media

Rank	Colony surface description
0	No wrinkling—a smooth colony surface
1	Single concentric undulation or contour
2	Radial ridges within a single concentric contour
3	Radial ridges within and beyond the single concentric contour
4	Additional striations on radial ridges within and beyond concentric contour

Glycerol in BPSA. Concentrations of 0, 1, 3, and 4% glycerol (vol/vol) were added to BPSA to examine its effect on surface wrinkling of colonies. Three plates of each glycerol concentration were streaked for single colonies with NCTC 13177, NCTC 10276, and BCC11. Wrinkling was ranked using the system shown in Table 1 at 48 h and 7 days.

At 48 h there was no wrinkling in strain 10276, but increasing the glycerol concentration increased wrinkling in strain 13177. Both strains produced colonies that were nonreflective at 96 h, and wrinkling was ranked 4 for all concentrations.

At 48 h the mucoid strain, BCC11, had smooth convex colonies at all glycerol concentrations. By 96 h the highest rank for wrinkling seen (2) was produced on the medium with the lowest concentration of glycerol (1%), while at the highest concentration there was no wrinkling. By 7 days, the mucoid strain produced only one large wrinkle per colony on the highest concentration of glycerol.

Evaluation of selective media. (i) *Burkholderia* species. Thirty clinical and 20 environmental strains of *B. pseudomallei* and 11 strains of *B. cepacia* were used to compare the sensitivities of BPSA and ASA. Overnight cultures of each strain were washed and diluted to a 10^4-CFU/ml suspension, and the plates were inoculated using a spiral plater. There were three replicates of each strain on BPSA and ASA, with the lower limits of detection being 2×10^1 CFU/ml. CFU were counted at 24 and 48 h. A further three plates each of BPSA and ASA were inoculated to produce single colony growth of each *B. pseudomallei* strain. The largest colonies on each plate were measured at ×1.5 magnification after 24 and 48 h of incubation and then measured again after 7 days. The colony counts and radial growth measurements on the two media were compared using paired *t* tests. The macroscopic appearance of the single colonies, including wrinkling of the colony surfaces, was observed at each of these time points. The degree of colony wrinkling was assessed using a ranking system (Table 1). This experiment was repeated once.

(a) *B. pseudomallei*. Colony counts of the reference strains of *B. pseudomallei* on BPSA were comparable ($P = 0.85$) to those on ASA, while the mucoid strain produced a significantly ($P < 0.05$) higher count on BPSA (Fig. 1). On BPSA, 98% of *B. pseudomallei* strains produced colony counts that were equivalent or higher ($P = 0.05$) than those on ASA (Table 2). At 24 h, seven clinical strains had not grown on ASA but produced counts of 10^4 organisms/ml on BPSA. The difference between the two media was reduced after 48 h of incubation.

The other most notable difference between BPSA and ASA was the significant increase ($P < 0.05$) in colony size of 36% of the *B. pseudomallei* strains on BPSA. All 50 strains produced

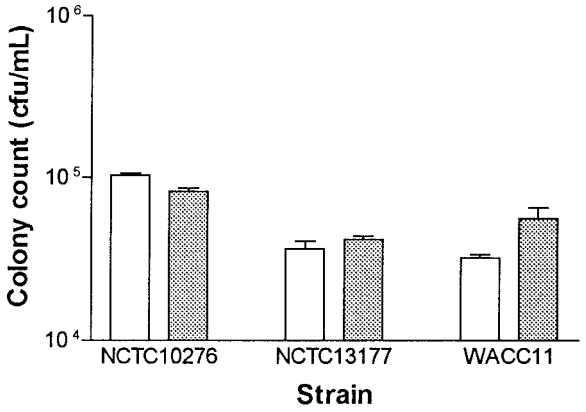


FIG. 1. Colony counts of *B. pseudomallei* strains after 48 h of incubation on ASA (white bars) and BPSA (gray bars).

TABLE 2. Growth of clinical strains and type strains of bacterial colonies at 48 h on ASA and BPSA

Bacterial species	Culture identification[b]	Growth[a] on:	
		ASA	BPSA
Escherichia coli	ATCC 25922	−	−
Enterococcus faecalis	ATCC 29212	−	−
Staphylococcus aureus	ATCC 25923	±	±
Streptococcus pyogenes	ATCC 19615	−	−
Pseudomonas alcaligenes	SSCC 18997	−	−
Pseudomonas fluorescens	SSCC 15298	−	±
Pseudomonas oryzihabitans	SSCC 17895	−	±
Pseudomonas pseudoalcaligenes[c]	SSCC 17344	−	±
Pseudomonas putida	SSCC 17676	±	−
Pseudomonas stutzen	SSCC 15643	−	−

[a] +, growth; ±, scanty growth; −, no growth.
[b] ATCC, American Type Culture Collection; SSCC, Sterile Site Culture Collection, PathCentre, Western Australia.
[c] Gentamicin resistant.

larger colonies on BPSA after 24 h of incubation, with 86% of strains being two to nine times larger. Of the three mucoid strains examined, two produced colonies that were 25% larger on BPSA. The difference in colony size on the two selective media decreased with time so that by 7 days only 60% of colonies were at least 10% larger on BPSA than on ASA.

One recently isolated clinical strain showed significantly ($P < 0.05$) more wrinkling on BPSA after 7 days. On ASA this strain did not wrinkle but presented as a convex smooth colony (ranked 0), while on BPSA it produced contours and some wrinkling (ranked 3). While there was no significant difference overall in the amount of wrinkling between colonies on each medium at 48 h, one-quarter of the strains was more wrinkled on BPSA than on ASA. This reflected a generally faster growth of colonies on BPSA.

While a variety of surface wrinkling patterns were observed on BPSA, all nonmucoid strains produced a characteristic flat, dry, mauve colony by 48 h. These colonies progressed over 7 days to become deep purple and highly wrinkled (Fig. 2). The exception to this was the one strain that only grew sparsely on ASA. This strain had distinct colonies that remained convex and absorbed little of the red pigment. Colonies of this strain were pink, circular, and lobate after 7 days. The persistently mucoid strain produced large wet colonies that had a blue-purple border and a pink-to-mauve center. Under UV illumination nonmucoid strains of *B. pseudomallei* produced yellow fluorescent colonies on BPSA. Fluorescence was fainter in mucoid colonies. Colonies grown on ASA did not fluoresce.

(b) *B. cepacia*. Four of the 11 strains of *B. cepacia* (from genomovars I, II, and III) were significantly ($t = 1.81$; $P = 0.009$) inhibited on BPSA compared to their growth on ASA at 24 and 48 h. One strain of genomovar I was totally inhibited on BPSA compared to growth on ASA. At 24 h, four strains had larger colonies on ASA than on BPSA, while three strains grew slightly larger on BPSA than on ASA.

In contrast to *B. pseudomallei*, colonies of *B. cepacia* did not wrinkle on BPSA. On this medium, *B. cepacia* formed circular, convex, transparent colonies with an entire edge by 24 h, and the colonies turned to a translucent pink color at 48 h. By 7 days *B. cepacia* produced opaque, dark pink, convex, lobed colonies. Seven strains of *B. cepacia* produced a similar fluorescence to *B. pseudomallei* under UV light. There was no fluorescence detected from the remaining four strains, which included both representatives of genomovar II.

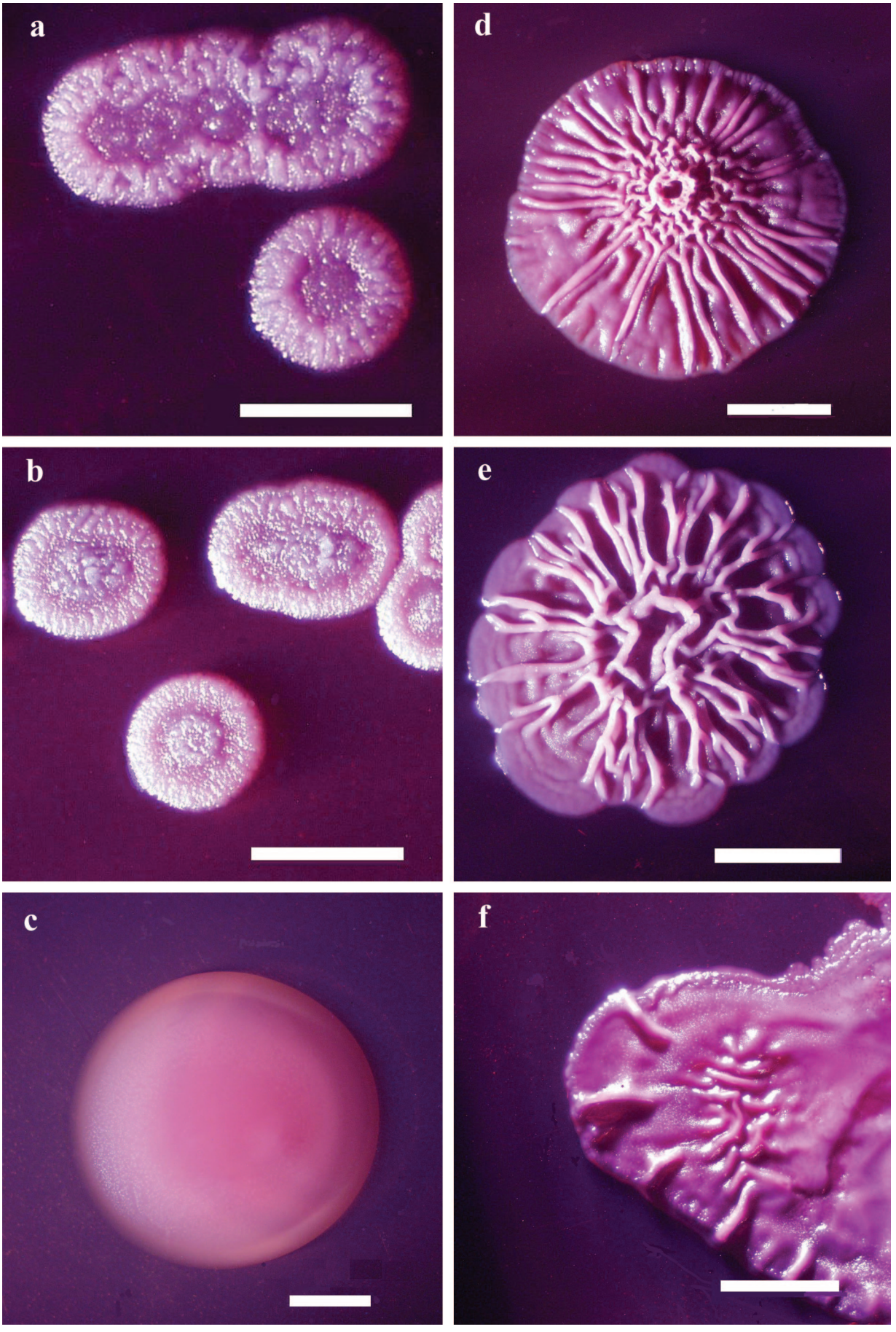
(ii) *Pseudomonas* species and other genera of clinical importance. To assess the selectivity of BPSA, overnight cultures of 51 strains of *P. aeruginosa* and 10 other clinical species (*E. coli*, *E. faecalis*, *Pseudomonas alcaligenes*, *P. fluorescens*, *Pseudomonas oryzihabitans*, *P. pseudoalcaligenes*, *P. putida*, *P. stutzeri*, *S. aureus*, and *S. pyogenes*) were prepared from overnight cultures. Three 10-μl aliquots of a 0.5 McFarland standard bacterial suspension were dropped onto two replicate plates of SMA, ASA, and BPSA. Growth of colonies from these drops was recorded for the three media as present, absent, or scanty at 24 and 48 h. Scanty growth was recorded when less than three colonies grew per aliquot or the colonies remained smaller than 1 mm. All bacteria tested grew on SMA within 24 h.

P. aeruginosa was significantly ($\chi^2 = 43.09$; $P < 0.0001$) inhibited on BPSA. Only 36% of strains grew by 48 h compared with 96% on ASA. All 51 strains grew on the SMA control plates within 24 h. All strains examined under UV fluoresced blue on BPSA.

While ASA proved to be more selective, the growth of *S. aureus*, *P. fluorescens*, *P. pseudoalcaligenes*, and *P. oryzihabitans* on BPSA was scant and did not appear until 48 h after inoculation (Table 2). There was a 9-$\log_{10}$ decrease in colony counts of gentamicin-resistant *P. aeruginosa* and *S. aureus* on all selective media used. On BPSA the *P. aeruginosa* strains fluoresced blue under UV illumination, while *E. faecalis*, *E. coli*, *S. pyogenes*, and *S. aureus* did not fluoresce.

In conclusion, this evaluation of selective media for the isolation of *B. pseudomallei* showed that the novel medium BPSA was better than ASA. The improvements included a reduced time to detection by increased numbers of colonies

FIG. 2. Colony morphology at 48 h (left) and 7 days (right) of *B. pseudomallei* grown on BPSA. (a and d) Strain NCTC 13177; (b and e) strain NCTC 10276; (c and f) the persistently mucoid strain WACC11. Bar = 1 mm.


a
d
b
e
c
f

and larger colony size and the recovery of strains of *B. pseudomallei* that are inhibited by ASA.

Although ASA performs well as a selective agar, it inhibits persistently mucoid strains of *B. pseudomallei*. It was previously noted that rough strains of *B. pseudomallei* grew on glycerol agar but that this medium was inhibitory to the smooth strain (7). In two of the three mucoid strains tested, BPSA produced larger colonies faster. As glycerol is a precursor for polyhydroxybutyrate synthesis, it is required in BPSA, but less glycerol was incorporated than is present in ASA. Crystal violet, which was inhibitory to mucoid strains, was excluded from BPSA. The isolation of mucoid variant strains from clinical specimens is uncommon at present; however, this may reflect the inhibitory effect of ASA on *B. pseudomallei* in nonsterile sites.

BPSA was highly selective against a variety of gram-positive and gram-negative bacterial species. *Pseudomonas* species, including some gentamicin-resistant strains of *P. aeruginosa*, were inhibited on BPSA. This is a feature that is expected to prove useful in inhibiting growth of *P. aeruginosa* commonly found as a contaminant in sputum specimens. There was moderate improvement in the inhibition of *B. cepacia* on BPSA from the genomovar collection tested. The combined inhibitory effect on bacterial species closely related to *B. pseudomallei* will assist the diagnostic laboratory by reducing the number of isolates that might otherwise be misidentified as *B. pseudomallei*. While the absence of wrinkling may be a key to the differentiation between *B. cepacia* and *B. pseudomallei*, there is still a need for further confirmatory tests such as PCR.

Diagnostic laboratories have come to depend on wrinkling as a morphological identification feature. BPSA provides large wrinkled colonies faster than ASA. This means that *B. pseudomallei* colonies are more visible and there is more bacterial growth for supplementary tests. This is particularly important for melioidosis, for public health control strategies that rely on rapid isolation of *B. pseudomallei* will also benefit from faster detection. This time reduction should also improve the quality of antibiotic treatment for patients. While public health investigations of melioidosis are hampered at present by delays in isolation of *B. pseudomallei* from nonsterile clinical sites, isolation of *B. pseudomallei* from environmental specimens such as soil and water takes even longer (3, 4). Given the current interest in a rapid public health response to a suspected deliberate biohazard release, there is a pressing need for an improved *B. pseudomallei* selective agar such as the novel medium we describe here.

Utilizing the above-mentioned morphological features, including UV fluorescence and color of colonies, means that less manipulation of suspect organisms is necessary in preliminary work. This should reduce exposure to aerosols of *B. pseudomallei*, which are known to have caused laboratory-acquired infection on at least two occasions (8).

The manufacture of BPSA is possible even in small clinical laboratories throughout the region where melioidosis is endemic. Preliminary assessments indicate that the shelf life is 8 weeks at 4°C. A more detailed evaluation of BPSA for the clinical diagnosis of melioidosis is now required.

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