



Murdoch
UNIVERSITY

MURDOCH RESEARCH REPOSITORY

<http://researchrepository.murdoch.edu.au>

This is the author's final version of the work, as accepted for publication following peer review but without the publisher's layout or pagination.

Howard, K. and Inglis, T.J.J. (2003) Novel Selective Medium for Isolation of Burkholderia pseudomallei. Journal of Clinical Microbiology, 41 (7). pp. 3312-3316.

<http://researchrepository.murdoch.edu.au/3309>

Copyright © 2003, American Society for Microbiology
It is posted here for your personal use. No further distribution is permitted.

Novel selective medium for the isolation of *Burkholderia pseudomallei*

K. Howard and T.J.J. Inglis

Division of Microbiology and Infectious Diseases, Western Australian Centre for
Pathology and Medical Research

Correspondence:

Dr K Howard, Division of Microbiology and Infectious Diseases, Western Australian
Centre for Pathology and Medical Research (PathCentre), Locked Bag 2009,
Nedlands WA 6909, AUSTRALIA.

Phone: +618 9346 3640

Fax: +618 9381 7139

Email: khoward@cyllene.uwa.edu.au

Keywords: *Burkholderia pseudomallei*, agar, medium

SUMMARY

For many years laboratory isolation of *Burkholderia pseudomallei*, the cause of the potentially fatal infection melioidosis, has relied on the use of Ashdown's selective agar (ASA). It has become apparent recently that ASA inhibits growth of some clinical isolates. We designed a new selective agar (*Burkholderia pseudomallei* selective agar; BPSA) to improve recovery of the more easily inhibited strains of *B. pseudomallei* and describe here the laboratory evaluation of that medium. Fifty strains of *B. pseudomallei*, 11 strains of *B. cepacia*, 51 strains of *Pseudomonas aeruginosa* and strains of other bacterial genera were used to determine the selectivity and sensitivity of BPSA. Most strains of *B. pseudomallei* produced larger colonies on BPSA than on ASA at 24 h. BPSA inhibited 4 of 11 strains of *Burkholderia cepacia* which all grew on ASA. BPSA should make recognition of *Burkholderia* species easier due to distinctive colony morphology. BPSA was also more inhibitory to *P. aeruginosa* than ASA. BPSA inhibited representative type strains of other bacterial species (*Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus pyogenes*). These results indicate that BPSA is a potential replacement for ASA. Use of BPSA in clinical diagnostic laboratories may confirm the anticipated high prevalence of undiagnosed subacute melioidosis in the main endemic areas of Northern Australia and South East Asia.

Laboratory diagnosis of acute septicaemic melioidosis is usually made by conventional blood culture techniques. In these circumstances a rich non-selective agar is adequate (7). However, isolation of *Burkholderia pseudomallei* is more difficult in clinical specimens from non-sterile body sites. Isolation from sputum is particularly difficult when low numbers of *B. pseudomallei* are present and there is an abundant commensal upper respiratory flora. The isolation of other non-fermentative 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 favoured medium for the isolation and presumptive identification of *B. pseudomallei* in melioidosis-endemic areas. *B. pseudomallei* produces highly wrinkled circular purple colonies on ASA by 48 h. A report of *B. pseudomallei* detection by rtPCR 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* (6). In that study, comparisons of ASA with a non-selective, 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 non-sterile sites since the original development of ASA. The aim of the present study was to develop a selective agar (BPSA; *Burkholderia pseudomallei* selective agar) that did not inhibit the persistently mucoid colony form of *B. pseudomallei* while maintaining selective activity against other bacteria.

Media development. Each of the ingredients of Ashdown's selective medium (trypticase peptone, 4% glycerol, 5 mg/L crystal violet, 50 mg/L neutral red and 4 mg/L gentamicin), Nile blue and 9 potential carbon sources were tested for inhibition of colony formation, colony size and wrinkling of colony surfaces by individual inclusion to a minimal media. In preliminary studies Nile blue proved to be an effective antibacterial supplement (6).

Primary carbon source. *B. pseudomallei* strains NCTC 13177 and BCC11, representative of non-mucoid and mucoid colony forms, respectively, three strains of *B. 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. 200 or 20 mg/L of each C source (maltose, glycerol, D-(-)- β -hydroxybutyric acid, salicin, succinic acid, sodium salicylate, D(+)-glucosamine, D-erythritol and isobutyric acid) was added to a minimal medium (K_2HPO_4 7 g/L, KH_2PO_4 2 g/L, $(NH_4)_2SO_4$ 1g/L, $MgSO_4$ 1 mL of 10 mg/mL). The control medium for this experiment contained no C source. Inoculum was prepared as 10 mL overnight cultures then centrifuged at 300 g for 15 min, the pellet was resuspended in 3 mL cold water for 10 min and centrifuged for a further 10 min. This wash step was repeated and the pellet was resuspended in minimal media with no carbon source. A 10 μ L inoculation of approximately 10^8 cfu/ml of each strain was put into 10 mL of pre-warmed (35°C) media. 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 colonies sizes of all C sources tested, while being slightly inhibitory to some *B. cepacia* strains.

Antibacterial supplements. To determine if crystal violet, neutral red or Nile blue are inhibitory to *B. pseudomallei* growth, 5, 100 and 20 mg/L of each, respectively, was added separately to standard methods agar (SMA) containing 2.67 mg/mL gentamicin. Inoculum of the two type strains (NCTC 13177 and 10276) and the mucoid strain (BCC11) were prepared as for the carbon source experiment and suspensions of approximately 10^4 organisms/mL water were spiral plated (Don Whitley, Shipley, UK) onto three replicate plates for each treatment. Colony counts were done at 24 h for each strain and at 48 h for the non-mucoid

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

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

BPSA preparation. BPSA comprised 23.5 g standard methods agar (BBL, USA), 4 g maltose (Sigma, St Louis, USA) and 100 mg neutral red (Sigma, St Louis, USA) in 1 L distilled water, sterilised at 134°C for 10 min (or 121°C for 15 min). The agar was allowed to cool to 40-45°C, then 20 mg/mL gentamicin (Sigma, St Louis, USA) and 1 mL of 20 g/L nile blue (dissolved in 1% DMSO) were added after filter sterilisation via a 0.8/0.2 µm membrane (Pall Corporation, MI, USA). Ten mL of glycerol (equivalent to 1%) (BDH, Merck P/L Australia) was added and the media 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, Western Australia, and included batch numbers 213164, 214750, 216753 and 220269.

Bacterial strains used to evaluate BPSA. The following strains were used: *B. pseudomallei* reference strains NCTC 10276, NCTC 13177, and 48 wild strains from clinical and environmental isolations, which included the persistently mucoid variant WACC11; 11 *B. cepacia* wild strains representative of each genomovar (T. Pitt, PHLS, Colindale, UK); 51 wild strains of *P. aeruginosa* and one wild strain each of *P. fluorescens*,

P. pseudoalcaligenes, *P. putida* and *P. stutzeri*, 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 and plated onto blood agar (BA) for a maximum of 3 d, then transferred to 10 mL trypticase soy broth (TSB) for 18 h incubation prior to use in the experiments. All incubations were in air at 35°C . Apart from 5 strains recently isolated, all strains had been stored for over 6 months prior to commencement of the present study.

Glycerol in BPSA. To examine the effect of glycerol on the surface wrinkling of colonies concentrations of 0, 1, 3, and 4 % (v/v) was added to BPSA. Three plates of each glycerol concentration were streaked for single colonies with NCTC 13177 and 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 glycerol concentration increased wrinkling in strain 13177. Both these non-mucoid strains had colonies that were non-reflective at 96 h, and wrinkling was ranked as 4 at 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 (2) was produced on the media with the lowest concentration of glycerol (1%) while at highest concentration the rank remained 0. By 7 days, the mucoid strain produced only one large wrinkle per colony on the highest concentration of glycerol.

EVALUATION OF SELECTIVE MEDIA

***Burkholderia* species.** 30 clinical and 20 environmental strains of *B. pseudomallei* and 11 strains of *B. cepacia* were used to compare the sensitivity of BPSA with 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 3 replicates of each strain on BPSA and ASA, with the lower limits of detection being 2.0×10^1 cfu/mL. Colony forming units were counted at 24 and 48 h. A further 3 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 1.5x magnification after 24 and 48 h incubation, and then measured again after 7 d. 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 surface, 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.

B. pseudomallei. Colony counts of the type strains of *B. pseudomallei* on BPSA were comparable ($p=0.85$) with those on ASA while the persistently mucoid strain produced a significantly ($p<0.05$) higher count on BPSA (Figure 1). On BPSA, 98% of *B. pseudomallei* strains produced equivalent or higher colony counts ($p=0.05$) than on ASA (Table 2). The exception was one clinical strain at 48 h. At 24 h, 7 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 incubation.

The other most notable difference between BPSA and ASA was the significant increase ($p<0.05$) in colony size of 36% of *B. pseudomallei* strains on BPSA. All 50 strains produced larger colonies on BPSA after 24 h incubation, with 86% of strains being 2 to 9 times larger. Of the 3 mucoid strains examined, 2 produced 25% larger colonies on BPSA. The difference in colony size on the two selective media decreased with time so that by 7 d 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 d. On ASA this strain did not wrinkle but presented as a convex smooth colony (ranked as 0), while on BPSA it produced contours and some wrinkling (ranked as 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.

Whilst a variety of surface wrinkling patterns was observed on BPSA, all non-mucoid strains produced a characteristic flat and dry, mauve coloured colony by 48 h. These colonies progressed over 7 d to become deep purple and highly wrinkled (Figure 2a and b). 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 d. The persistently mucoid strain produced large wet colonies that had a blue/purple border and a pink to mauve centre (Figure 2c). Under UV

illumination non-mucoid strains of *B. pseudomallei* produced yellow fluorescent colonies on BPSA. Fluorescence was fainter in mucoid colonies. Colonies grown on ASA did not fluoresce.

***B. cepacia*.** Four of the eleven strains of *B. cepacia* (from genomovar 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 compared to growth on ASA. At 24 h, four strains had larger colonies on ASA than on BPSA, while 3 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 turned to a translucent pink colour at 48 h. By 7 d *B. cepacia* produced opaque and 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.

***Pseudomonas* species and other genera of clinical importance.** To assess the selectivity of BPSA, overnight cultures of 51 strains of *Pseudomonas aeruginosa* and 10 other clinical species (*Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas alcaligenes*, *P. fluorescens*, *P. oryzihabitans*, *P. pseudoalcaligenes*, *P. putida*, *P. stutzeri*, *Staphylococcus aureus* and *Streptococcus pyogenes*) were prepared from overnight cultures. Three 10 μ L aliquots of a 0.5 McFarland's 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 3 colonies grew per inoculation 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 *Staphylococcus aureus* and *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 the novel medium BPSA was better than Ashdown's selective agar medium. The improvements included a reduced time to detection by increased numbers of colonies and larger colony size, and the recovery of strains of *B. pseudomallei* 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 this medium was inhibitory to the smooth strain (8). In 2 out of the 3 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 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 non-sterile sites. The only persistently mucoid strains isolated to date in Australia have been recovered on non-selective agar in clinical diagnostic use.

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 a 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 larger 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 in melioidosis where 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 non-sterile 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.

Utilising the above mentioned morphological features including UV fluorescence and colour 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 (9).

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

This work was sponsored by the CRC for Water Quality and Treatment which is supported under the Australian Government's Cooperative Research Centres Program. We gratefully acknowledge the gift of bacterial isolates used in this study by Professor B. J. Currie and Dr G. Lum, Northern Territory; Dr R. Norton, Queensland; Dr T. Pitt, UK and Professor S. D. Puthuchery, Malaysia. We thank our colleagues at PathCentre, particularly R. Hunter. We also thank Professor Tom Riley of University of Western Australia for reviewing this manuscript and Dr Paul Rigby for assistance with photography.

REFERENCES

1. **Ashdown, L. R.** 1979. An improved screening technique for isolation of *Pseudomonas pseudomallei* from clinical specimens. *Pathology* **11**:293-297.
2. **Brook, M. D., B. Currie, and P. M. Desmarchelier.** 1997. Isolation and identification of *Burkholderia pseudomallei* from soil using selective culture techniques and the polymerase chain reaction. *J. Appl. Microbiol.* **82**:589-596.
3. **Inglis, T. J.J., S. C. Garrow, C. Adams, M. Henderson, and M. Mayo.** 1998. Dry-season outbreak of melioidosis in Western Australia. *Lancet* **352**:1600.
4. **Inglis, T. J.J., S. C. Garrow, C. Adams, M. Henderson, M. Mayo, and B. J. Currie.** 1999. Acute melioidosis outbreak in Western Australia. *Epidemiol. Infect.* **123**:437-443.
5. **Inglis, T. J. J., B. J. Mee, and B. J. Chang.** 2000. The environmental microbiology of melioidosis. *Rev. Med. Microbiol.* **12(1)**:13-20.
6. **Inglis, T. J. J., and R. J. Hunter.** 2000. New selective medium for environmental *Burkholderia pseudomallei*. Poster, Annual Scientific Meeting, American Society for Microbiology, Los Angeles, CA.
7. **Pitt, T.** 1998. *Pseudomonas, Burkholderia* and related genera, p. 1120-1127 *In* A. Balows and B. I. Duerden (ed.), *Topley and Wilson's Microbiology and Microbial Infections*, vol. 2, 9th ed. Oxford University Press, New York.
8. **Rogul, M., and S. R. Carr.** 1972. Variable ammonia production among smooth and rough strains of *Pseudomonas pseudomallei*: resemblance to bacteriocin production. *J. Bacteriol.* **112**:372-380.
9. **Schlech, W. F., J. B. Turchick, R. E. Westlake, G. C. Klein, J. D. Band, and R. E. Weaver.** 1981. Laboratory-acquired infection with *Pseudomonas pseudomallei* (melioidosis). *New England J. Medicine* **305**:1133-1135.

Table 1. Ranking system for surface wrinkling of single colonies of *Burkholderia 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

Table 2. Growth (+), scanty growth (+/-) or no growth (-) of clinical strains and type strains of bacterial colonies at 48 h on Ashdown's selective agar (ASA) and *Burkholderia pseudomallei* selective agar (BPSA).

Bacterial species	Culture identification	ASA	BPSA
<i>Escherichia coli</i>	ATCC 25922	-	-
<i>Enterococcus faecalis</i>	ATCC 29212	-	-
<i>Staphylococcus aureus</i>	ATCC 25923	+/-	+/-
<i>Streptococcus pyogenes</i>	ATCC 19615	-	-
<i>Pseudomonas alcaligenes</i>	SSCC 18997	-	-
<i>P. fluorescens</i>	SSCC 15298	-	+/-
<i>P. oryzihabitans</i>	SSCC 17895	-	+/-
<i>P. pseudoalcaligenes</i> *	SSCC 17344	-	+/-
<i>P. putida</i>	SSCC 17676	+/-	-
<i>P. stutzeri</i>	SSCC 15643	-	-

* Gentamicin resistant

ATCC is the American Type Culture Collection

SSCC is the Sterile Site Culture Collection, PathCentre, Western Australia

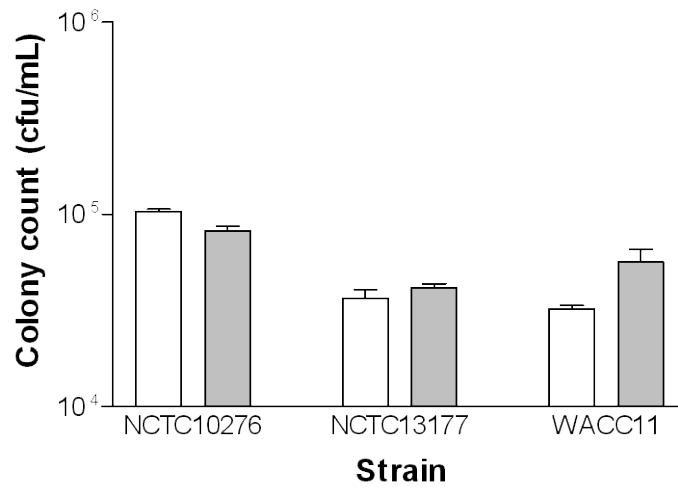


Figure 1. Colony counts of *Burkholderia pseudomallei* strains after 48 h incubation on
 □ Ashdown's selective agar (Ashdown, 1979) and ■ BPSA (*Burkholderia pseudomallei*
 selective agar).

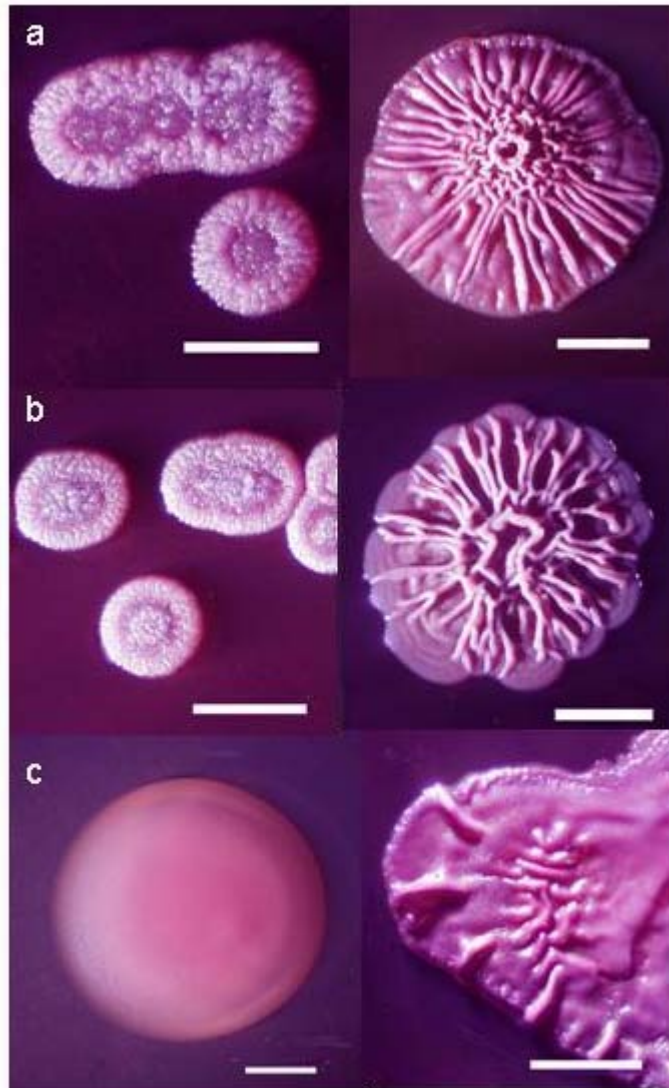


Figure 2. Colony morphology at 48 h (left) and 7 d (right) of *Burkholderia pseudomallei* on *B. pseudomallei* selective media (BPSA). a) type strain NCTC 13177, b) type strain NCTC 10276, and c) the persistently mucoid strain WACC11. Bar = 1 mm.