Evaluation of consensus method for the culture of Burkholderia pseudomallei in soil samples from Laos
David A.B. Dance, Michael Knappik, Sabine Dittrich, Viengmon Davong, Joy Silisouk, Manivanh Vongsouvath, Sayaphet Rattanavong, Alain Pierret, Paul Newton, Premjit Amornchai, et al.

To cite this version:
David A.B. Dance, Michael Knappik, Sabine Dittrich, Viengmon Davong, Joy Silisouk, et al.. Evaluation of consensus method for the culture of Burkholderia pseudomallei in soil samples from Laos. Wellcome Open Research, F1000Research, 2018, 3, pp.132. 10.12688/wellcomeopenres.14851.2. hal-01974451

HAL Id: hal-01974451
https://hal.sorbonne-universite.fr/hal-01974451
Submitted on 8 Jan 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Evaluation of consensus method for the culture of *Burkholderia pseudomallei* in soil samples from Laos [version 2; referees: 2 approved]

David A.B. Dance¹,²,³, Michael Knappik¹,⁴, Sabine Dittrich¹,²,⁵, Viengmon Davong¹, Joy Silisouk¹, Manivanh Vongsouvath¹, Sayaphet Rattanavongⁱ¹, Alain Pierretⁱ,⁶ Paul N. Newton¹,², Premjit Amornchai⁷, Vanaporn Wuthiekanunⁱ⁷, Sayan Langla⁷, Direk Limmathurotsakulⁱ²,⁷

¹Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit, Mahosot Hospital, Vientiane, Lao People’s Democratic Republic
²Centre for Tropical Medicine and Global Health, University of Oxford, Oxford, OX3 7FZ, UK
³Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London, WC1E 7HT, UK
⁴Médecins Sans Frontières, Maputo, Mozambique
⁵Foundation for Innovative Diagnostics, Geneva, Switzerland
⁶Institut de Recherche pour le Développement (IRD), iEES-Paris (IRD, Sorbonne Universités, UPMC Univ Paris 06, CNRS, INRA, UPEC, Université Paris Diderot), Department of Agricultural Land Management (DAlAM), Vientiane, Lao People’s Democratic Republic
⁷Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, 10400, Thailand

Abstract

**Background:** We have previously shown that PCR following enrichment culture is the most sensitive method to detect *Burkholderia pseudomallei* in environmental samples. Here we report an evaluation of the published consensus method for the culture of *B. pseudomallei* from Laos soil in comparison with our conventional culture method and with PCR with or without prior broth enrichment.

**Methods:** One hundred soil samples were collected from a field known to contain *B. pseudomallei* and processed by: (i) the conventional method, (ii-iii) the consensus method using media prepared in either Laos or Thailand, and (iv) the consensus method performed in Thailand, as well as by (v) PCR following direct extraction of DNA from soil and (vi) PCR following broth pre-enrichment.

**Results:** The numbers of samples in which *B. pseudomallei* was detected were 42, 10, 7, 6, 6 and 84, respectively. However, two samples were positive by the consensus method but negative by conventional culture, and one sample was negative by PCR following enrichment although *B. pseudomallei* was isolated by the conventional culture method.

**Conclusions/Discussion:** The results show that no single method will detect all environmental samples that contain *B. pseudomallei*. People conducting environmental surveys for this organism should be aware of the possibility of false-negative results using the consensus culture method. An approach that entails screening using PCR after enrichment, followed by the evaluation of a
range of different culture methods on PCR-positive samples to determine which works best in each setting, is recommended.

**Keywords**
Burkholderia pseudomallei, melioidosis, soil, environmental samples, culture, detection, Laos, Lao PDR

**Corresponding author:** David A.B. Dance (David.d@tropmedres.ac)

**Author roles:**
- **Dance DAB:** Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Project Administration, Resources, Supervision, Validation, Writing – Original Draft Preparation, Writing – Review & Editing
- **Knappik M:** Formal Analysis, Investigation, Methodology, Writing – Review & Editing
- **Dittrich S:** Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Supervision, Writing – Review & Editing
- **Davong V:** Investigation, Supervision, Writing – Review & Editing
- **Silisouk J:** Investigation, Writing – Review & Editing
- **Vongsouvath M:** Investigation, Supervision, Writing – Review & Editing
- **Rattanavong S:** Investigation, Writing – Review & Editing
- **Pierret A:** Formal Analysis, Investigation, Methodology, Supervision, Writing – Review & Editing
- **Newton PN:** Funding Acquisition, Resources, Writing – Review & Editing
- **Amornchai P:** Investigation, Methodology, Supervision, Writing – Review & Editing
- **Langla S:** Investigation, Writing – Review & Editing
- **Limmathurotsakul D:** Conceptualization, Formal Analysis, Funding Acquisition, Methodology, Project Administration, Resources, Supervision, Validation, Writing – Review & Editing

**Competing interests:** No competing interests were disclosed.

**Grant information:** This work was supported by the Wellcome core grant to the Mahidol-Oxford Tropical Medicine Research network (106698/Z/14/Z) and by a seed award from the Lee Ka Shing Foundation to the University of Oxford (SM40).

**Copyright:** © 2018 Dance DAB et al. This is an open access article distributed under the terms of the Creative Commons Attribution Licence, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**How to cite this article:** Dance DAB, Knappik M, Dittrich S et al. *Evaluation of consensus method for the culture of Burkholderia pseudomallei in soil samples from Laos [version 2; referees: 2 approved]* Wellcome Open Research 2018, 3:132 (https://doi.org/10.12688/wellcomeopenres.14851.2)

**First published:** 15 Oct 2018, 3:132 (https://doi.org/10.12688/wellcomeopenres.14851.1)
Amendments from Version 1

We are grateful to the referees for taking the time to help us improve our paper. The main changes in version 2 are as follows:

1. An explanation of how confidence intervals were calculated has been added.
2. Additional possible explanations for the difference in performance of the consensus method between this and the previous study have been added.
3. A paragraph summarising the limitations of the study identified by the referees has been added.

See referee reports

Introduction

Melioidosis, or infection with *Burkholderia pseudomallei*, is an important but under-recognised public health problem throughout the tropics\(^1\). The causative agent is a Gram-negative saprophyte found in soil and surface water in endemic areas. There have been numerous studies describing the detection of *B. pseudomallei* from soil\(^2,3\). These studies have used a wide range of both culture and molecular approaches. In 2013, an attempt was made to standardise these approaches, and a culture method, based on a technique that had a comparable sensitivity to semiquantitative culture on solid media during a small-scale evaluation in northeast Thailand\(^4\), was published and proposed as a consensus methodology\(^5\). This method, which uses enrichment culture and is thus only qualitative, has not yet been formally evaluated elsewhere. During studies of the seasonal variation of the distribution of *B. pseudomallei* in a rice paddy in northern Laos, we noticed that enrichment cultures often failed to isolate *B. pseudomallei* even when it was isolated from the same sample on solid media\(^6\). This led to a formal comparison of the consensus method on Lao soil with other culture and molecular methods. The results of the comparison of the molecular methods with culture on Lao soil and water samples have already been published\(^7\), and this paper will focus on a comparison of the performance of the consensus soil method with other methods.

| Method                                  | Description                                                                 | Reference |
|-----------------------------------------|-----------------------------------------------------------------------------|-----------|
| i. Conventional semiquantitative culture (ASH) | 100 g soil - Semi-quantitative culture on Ashdown agar in LOMWRU using the method used previously in soil surveys in Laos. | 3         |
| ii. Consensus method (CON-VTE).         | 10 g soil - Culture by the ‘consensus method’ in LOMWRU using Ashdown agar prepared in LOMWRU. | 33        |
| iii. Consensus method (CON-VTE/BKK).    | 10 g soil - Culture by the consensus method in LOMWRU, using Ashdown agar prepared in MORU and shipped to Laos (in order to help determine whether differences in media performance or laboratory technique might account for differences in the performance of the consensus method in LOMWRU). | 33        |
| iv. Consensus method (CON-BKK).         | 10 g soil - Culture by the consensus method performed in MORU                | 33        |
| v. Direct PCR (DS/qPCR)                 | 0.5 g soil - PCR following direct DNA extraction from soil in LOMWRU        | 13        |
| vi. PCR following enrichment (ES/qPCR)  | 20 g soil - PCR following broth enrichment culture in LOMWRU                | 13        |

Methods

Sample collection

Soil sampling was performed during the dry season (April 2013) in a rice paddy near the village of Ban Nabaone, Vientiane Province, Laos (18°22’51.4”N, 102°25’27.8”E, altitude 195 m), as previously described\(^1\). In brief, samples were collected at two depths (30 cm and 60 cm) at 50 points within a section of the field previously determined to have the highest positivity rates for *B. pseudomallei* by culture\(^4\) (total samples = 100).

Written permission to collect the samples was obtained from the village office on the authority of the Director of Mahosot Hospital, but only oral informed consent was obtained from the farmers concerned on the advice of the village office. The samples were placed in sterile plastic bags in an insulated box in the shade and maintained at ambient temperature during transport and subsequent manipulation. Once received in the Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit (LOMWRU) laboratory in Vientiane, each soil sample was split into six representative sub-samples using the Japanese slab cake method\(^8\). One sub-sample of each sample was then shipped to the Mahidol-Oxford Tropical Medicine Research Unit (MORU) laboratory in Bangkok.

Sample processing

In order to avoid any variations occurring prior to processing, the processing was co-ordinated between the LOMWRU and MORU laboratories so that all methods started simultaneously. One sub-sample from each sample was processed in one of six ways (Table 1). Samples were collected within 24 h of each other and subsampling was performed up to ~72 h after collection. Processing of all samples was started on the same day, ~120 h after collection. The methods used were as described in the respective references but are summarised briefly below.

1. **Conventional semiquantitative culture (ASH)**. 100 g of each soil sample was added to 100 ml of sterile water and suspended by vigorous agitation. The sample was then left to settle overnight. The following day, 2 × 10 μl, 2 × 100 μl and 1 × 500 μl volumes of the supernatant were inoculated onto Ashdown agar plates (containing Trypticase soy agar with
4% glycerol, crystal violet 5 mg/l, neutral red 50 mg/l and gentamicin 8 mg/l34) and 1 ml into 10 ml TBSS-C50, prepared in MORU (containing threonine-basal salt solution (TBSS) plus colistin 50 mg/l)32. The inoculum was then spread evenly to cover the entire surface of each agar plate, and all cultures were incubated at 40–42°C in air. The TBSS-C50 broth was incubated for 48 h and then 10 μl from the surface was subcultured onto an Ashdown agar plate. Ashdown plates were read on days 2, 3 and 4 of incubation.

ii, iii and iv. Consensus method (CON-VTE, CON-VTE/BKK and CON-BKK). 10 ml TBSS-C50 broth was added to 10 g soil and was vortex-mixed for 30 sec before being incubated at 40–42°C in air for 48 h. The surface of the broth (10 μl) was subcultured onto both an Ashdown plate prepared in LOMWRU and an Ashdown plate prepared in MORU. The plates were incubated at 40–42°C in air and read as above. The same method was used in MORU using only locally prepared media.

A single tube of TBSS-C50 was inoculated with a known B. pseudomallei clinical isolate and incubated along with the samples as a positive control for the culture methods.

All suspected isolates were screened by agglutination with a latex agglutination reagent specific for the 200 kDa extracellular polysaccharide of B. pseudomallei and tested for susceptibility to co-amoxiclav and resistance to colistin. All presumptive isolates were confirmed as B. pseudomallei by qPCR36 and 10% of isolates were also confirmed by API 20NE.

v and vi. Molecular detection (DS/qPCR and ES/qPCR). The molecular methods used in this study were based on the methods of Kaestli et al.2 and are described in detail in Knappik et al.2015. In brief, DNA was extracted directly from ~0.5 g of soil or after enrichment culture. Enrichment was performed as follows: soil was homogenized in modified Ashdown’s broth, shaken for 2 h at 240 rpm, and then incubated at 37°C for 22 h. The liquid phase was decanted and centrifuged (700 × g, 2 min), and the supernatant was removed and aurintricarboxylic acid was added2. After final centrifugation (45 min, 4,000 × g), DNA was extracted from the pellet. DNA was extracted using the MoBio PowerSoil® DNA Isolation kit2 and 4 μl of soil DNA was used to amplify the orf2 stretch of the TTS1 gene of B. pseudomallei36. To reduce the effect of inhibitors, 400 ng/μl of bovine serum albumin (BSA, New England Biolab, USA) was added2.

Statistical analysis
The sensitivity of each method was defined by comparing yield against the cumulative yield for all six methods and the confidence intervals for sensitivities were estimated by using the ci command in STATA. McNemar’s exact test was used to compare the sensitivity of two methods. Statistical analyses were performed using STATA/MP version 14.2 (College Station, Texas, United States).

Results and discussion
The proportion of the 100 samples in which B. pseudomallei was detected by each method is shown in Figure 1. Overall, B. pseudomallei was detected in 85 samples by at least one method.

The lowest sensitivities (7% [6/85]; 95% CI: 2.6-14.7%) were obtained using the consensus method in MORU and by PCR following direct extraction of DNA from soil. We have previously reported the low yield of direct DNA extraction from soil13 and this will not be discussed further here. The sensitivity of the consensus method conducted in LOMWRU using locally made media (12% [10/85]; 95%CI 5.8-20.6%) or media prepared in MORU (8% [7/85]; 95%CI 3.4-16.2) was slightly higher than the sensitivity of the consensus method in MORU although this did not achieve significance (p=0.29 and p>0.99, respectively). The sensitivity of the conventional culture method (49% [42/85]; 95%CI 38.4-60.5%) was significantly higher than

Figure 1. Proportion of samples that yielded B. pseudomallei by method. The number of the 100 samples in which B. pseudomallei was detected by each method is shown. Abbreviations: ASH, conventional semi-quantitative culture; CON-VTE, consensus method in LOMWRU using locally made media; CON-VTE/BKK, consensus method in LOMWRU using Ashdown agar made in MORU; CON-BKK, consensus method performed in MORU; DS/qPCR, PCR following direct extraction of DNA from soil; ES/qPCR, PCR following broth enrichment culture.
that of the three consensus methods (all p values<0.001). The sensitivity of PCR following an enrichment culture step (98.8% [84/85]; 95%CI 93.6-99.9) was significantly higher than that of the conventional culture method and all other methods (all p values<0.001). (Figure 1). There were, however, two samples in which B. pseudomallei was not detected by conventional culture but in which it was isolated using one of the consensus methods (in one case only in MORU and in the other in all three variations). There was also a single sample from which B. pseudomallei was isolated by conventional culture but in which B. pseudomallei was not detected by any other method, including PCR following enrichment culture.

We and others have already demonstrated that PCR following enrichment culture is currently the most sensitive method for the detection of B. pseudomallei in both soil and surface water samples. However, in this study we showed that the consensus method was significantly less sensitive than the conventional culture method when using soil from a field in Ban Nabone, Vientiane Province, Laos, some 560 km away from Ubon Ratchathani in Thailand where the consensus method was originally evaluated and found to have high sensitivity. This difference between the conventional culture method and the consensus method could not be explained by variations in culture media or in the experience of the staff reading the culture plates, as we controlled for all of these factors. The reasons for this variation in the sensitivity of the consensus method on soil from different regions is not known, but could include differences in the numbers of B. pseudomallei present in the soil and the amounts of soil processed in the different methods, differences in the range of competing flora present resulting in overgrowth of B. pseudomallei in the enrichment broth, differences in the soil type (for example clay as opposed to sandy soil), and possibly the activity of lytic bacteriophages during the enrichment culture step. The different amounts of soil used in the various methods is also likely to influence the sensitivity of each method. Others have also reported finding that B. pseudomallei was not isolated from broth cultures despite its apparent enrichment as evidenced by PCR. The possibility of the organism being in a ‘viable but non-cultivable state’ has been discussed, but this would not explain the apparent amplification of the organism by enrichment culture when comparing the results of direct extraction and PCR with those of enrichment culture and PCR.

There are a number of potential limitations of this study. First, it was conducted in only a single location and it is thus impossible to say how widespread is the issue of sub-optimal sensitivity of the consensus method. However, the fact that it fails to detect a substantial proportion of B. pseudomallei-positive samples in at least one location should alert other researchers to this possibility wherever they are working. Secondly, the uneven distribution of B. pseudomallei in soil could have accounted for some sub-samples not containing the organism, although we attempted to minimise the risk of this by conducting the sub-sampling using the Japanese slab-cake method. Thirdly, the culture methods are dependent on highly skilled technicians and detection of B. pseudomallei depends on them being able to recognise colonies with the appearance of B. pseudomallei, meaning that atypical (e.g. moist or mucoid) colonies might be missed, although this is the case with both culture methods. Fourthly, although it is generally agreed that the orf2 stretch of the TTS1 gene is highly specific for B. pseudomallei, there may be other as yet uncharacterised organisms closely related to B. pseudomallei in the environment that could have given false-positive PCR reactions.

Whilst preliminary and requiring confirmation in other sites, the implication of these findings is that anyone using the consensus method alone might fail to isolate B. pseudomallei in a given area, especially if only a small number of samples are tested. Unfortunately, the conventional method is time-consuming, labour-intensive, and requires highly trained and experienced staff to detect small numbers of colonies of B. pseudomallei in the midst of a range of competing flora. These results also demonstrated that no method is perfect in detecting B. pseudomallei in environmental samples. Despite the higher overall sensitivity of the conventional culture method, there were still two samples from which B. pseudomallei was isolated using the consensus method but which were culture-negative by the conventional method, just as there was one sample from which B. pseudomallei was isolated by the conventional method despite not being detected by PCR following enrichment.

The development of the consensus method was intended to try to standardise the work being done by many research groups to determine the global distribution of B. pseudomallei in the environment. Although the consensus method has been successful in isolating B. pseudomallei from soil in many regions in Thailand, we believe that it is important that other researchers in this field are made aware that it appears not to have equivalent sensitivity everywhere. Until we understand the reasons why the consensus method has a higher sensitivity in some places than others, we caution others conducting such studies that a failure to isolate B. pseudomallei from the environment using the consensus method does not mean that it is not present. Based on our own experience, we suggest that perhaps the most logical approach to looking for B. pseudomallei in a new environment would be to use enrichment culture followed by PCR as a screening method, and then to attempt a range of culture methods on PCR-positive samples until one is found that is able to isolate B. pseudomallei.

Data availability

Open Science Framework: Evaluation of consensus method for detection of B. pseudomallei in soil, https://doi.org/10.17605/OSF.IO/35GHQ

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).
Grant information

This work was supported by the Wellcome core grant to the Mahidol-Oxford Tropical Medicine Research network (106698/Z/14/Z) and by a seed award from the Lee Ka Shing Foundation to the University of Oxford (SM40).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

We are grateful to all staff at the Microbiology Laboratory, Mahosot Hospital, Vientiane, Laos who helped with the cultures and the farmers who granted permission for digging their land. We are grateful to Weerawat Wongasa for media preparation. We are also grateful to the Minister of Health, and the Director of the Curative Department, Ministry of Health, for their support for the work of the Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit.

References

1. Limmathurotsakul D, Golding N, Dance DA, et al.: Predicted global distribution of Burkholderia pseudomallei and burden of melioidosis. Nat Microbiol. 2016; 1: 15008. PubMed Abstract | Publisher Full Text
2. Kaestli M, Mayo M, Harrington G, et al.: Sensitive and specific molecular detection of Burkholderia pseudomallei, the causative agent of melioidosis, in the soil of tropical northern Australia. Appl Environ Microbiol. 2007; 73(21): 6891–7. PubMed Abstract | Publisher Full Text | Free Full Text
3. Rattanavong S, Wuthiekanun V, Langsa T, et al.: Randomized soil survey of the distribution of Burkholderia pseudomallei in rice fields in Laos. Appl Environ Microbiol. 2011; 77(2): 532–6. PubMed Abstract | Publisher Full Text | Free Full Text
4. Trung TT, Hetzer A, Göhler A, et al.: Highly sensitive direct detection and quantification of Burkholderia pseudomallei bacteria in environmental soil samples by using real-time PCR. Appl Environ Microbiol. 2011; 77(18): 6486–94. PubMed Abstract | Publisher Full Text | Free Full Text
5. Vongphayloth K, Rattanavong S, Moore CE, et al.: Burkholderia pseudomallei detection in surface water in southern Laos using Moore’s swabs. Am J Trop Med Hyg. 2012; 86(5): 872–7. PubMed Abstract | Publisher Full Text | Free Full Text
6. Wuthiekanun V, Mayxay M, Chierakul W, et al.: Detection of Burkholderia pseudomallei in soil within the Lao People’s Democratic Republic. J Clin Microbiol. 2005; 43(2): 923–4. PubMed Abstract | Publisher Full Text | Free Full Text
7. Brook MD, Currie B, Desmaraischer PM: Isolation and identification of Burkholderia pseudomallei from soil using selective culture techniques and the polymerase chain reaction. J Appl Microbiol. 1997; 82(6): 589–96. PubMed Abstract | Publisher Full Text | Free Full Text
8. Chen YS, Lin HH, Mu JJ, et al.: Distribution of melioidosis cases and viable Burkholderia pseudomallei in soil: evidence for emerging melioidosis in Taiwan. J Clin Microbiol. 2010; 48(4): 1432–4. PubMed Abstract | Publisher Full Text | Free Full Text
9. Ellison DW, Baker HJ, Mariappan M: Melioidosis in Malaysia. I. A method for isolation of Pseudomonas pseudomallei from soil and surface water. Am J Trop Med Hyg. 1969; 18(5): 694–7. PubMed Abstract | Publisher Full Text
10. Hantrakun V, Rongkard P, Ouyoucha M, et al.: Soil Nutrient Depletion Is Associated with the Presence of Burkholderia pseudomallei. Appl Environ Microbiol. 2016; 82(4): 7068–92. PubMed Abstract | Publisher Full Text | Free Full Text
11. Jilani MS, Robayet JA, Mohiuddin M, et al.: Burkholderia pseudomallei: Its Detection in Soil and Seroprevalence in Bangladesh. PLoS Negl Trop Dis. 2016; 10(1): e0004301. PubMed Abstract | Publisher Full Text | Free Full Text
12. Kaestli M, Mayo M, Harrington G, et al.: Landscape changes influence the occurrence of the melioidosis bacterium Burkholderia pseudomallei in soil in northern Australia. PLoS Negl Trop Dis. 2009; 3(1): e3634. PubMed Abstract | Publisher Full Text | Free Full Text
13. Knappik M, Dance DA, Rattanavong S, et al.: Evaluation of Molecular Methods To Improve the Detection of Burkholderia pseudomallei in Soil and Water Samples from Laos. Appl Environ Microbiol. 2015; 81(11): 3726–7. PubMed Abstract | Publisher Full Text | Free Full Text
14. Lau SK, Chan SY, Curreen SO, et al.: Burkholderia pseudomallei in soil samples from an oceanarium in Hong Kong detected using a sensitive PCR assay. Emerg Microbes Infect. 2014; 3(10): e69. PubMed Abstract | Publisher Full Text | Free Full Text
15. Limmathurotsakul D, Wuthiekanun V, Chantratita N, et al.: Burkholderia pseudomallei is spatially distributed in soil in northeast Thailand. PLoS Negl Trop Dis. 2010; 4(6): e694. PubMed Abstract | Publisher Full Text | Free Full Text
16. Musa HI, Hassan L, Shamsuddin ZH, et al.: Physicochemical Properties Influencing Presence of Burkholderia pseudomallei in Soil from Small Ruminant Farms in Peninsular Malaysia. PLoS One. 2016; 11(9): e0162348. PubMed Abstract | Publisher Full Text | Free Full Text
17. Ngamsang R, Potsap C, Boonmeer A, et al.: The contribution of soil physicochemical properties to the presence and genetic diversity of Burkholderia pseudomallei. Southeast Asian J Trop Med Public Health. 2015; 46(1): 38–50. PubMed Abstract
18. Palasaitien S, Lertsirinovarak R, Royros P, et al.: Soil physicochemical properties related to the presence of Burkholderia pseudomallei. Trans R Soc Trop Med Hyg. 2008; 102 Suppl 1: S5–9. PubMed Abstract | Publisher Full Text
19. Prakash A, Thavaselvam D, Kumar A, et al.: Isolation, identification and characterization of Burkholderia pseudomallei from soil of coastal region of India. SpringerPlus. 2014; 3: 438. PubMed Abstract | Publisher Full Text | Free Full Text
20. Sermawan RW, Royros P, Kohkhum N, et al.: Direct detection of Burkholderia pseudomallei and biological factors in soil. Trans R Soc Trop Med Hyg. 2015; 109(7): 462–8. PubMed Abstract | Publisher Full Text
21. Smith MD, Wuthiekanun V, Walsh AL, et al.: Quantitative recovery of Burkholderia pseudomallei from soil in Thailand. Trans R Soc Trop Med Hyg. 1995; 89(5): 488–90. PubMed Abstract | Publisher Full Text
22. Sousa VVK, Segovia JFO, Martins POJ, et al.: Preliminary molecular studies of the first report of Burkholderia pseudomallei isolation from soil collected in the Amapá State, in Northern Brazil. Int J Biol. 2016; 8(1): 9–16. Publisher Full Text
23. Strauss JM, Groves MG, Mariappan M: Melioidosis in Malaysia. II. Distribution of Pseudomonas pseudomallei in soil and surface water. Am J Trop Med Hyg. 1969; 18(5): 698–702. PubMed Abstract | Publisher Full Text
24. Strauss JM, Jason S, Mariappan M: Pseudomonas pseudomallei in soil and surface water of Sabah, Malaysia. Med J Malaya. 1967; XXII(1): 31–2. Reference Sources
25. Suebrasri T, Wang-ngarm S, Chareonsudja P, et al.: Seasonal variation of soil environmental characteristics affect the presence of Burkholderia pseudomallei in Phuket, Thailand. Afr J Microbiol Res. 2013; 7(19): 1940–5. Publisher Full Text
26. Thomas AD: The isolation of Pseudomonas pseudomallei from soil in Northern Queensland, Aust Vet J. 1977; 53(9): 408. PubMed Abstract | Publisher Full Text
27. Thomas AD, Forbes-Faulkner JC: Persistence of Pseudomonas pseudomallei in soil. Aust Vet J. 1981; 57(11): 535–6. PubMed Abstract | Publisher Full Text
28. Trung TT, Hetzer A, Topfstedt E, et al.: Improved culture-based detection and quantification of Burkholderia pseudomallei from soil. Trans R Soc Trop Med Hyg. 2011; 105(6): 346–51. PubMed Abstract | Publisher Full Text
29. Wuthiekanun V, Smith MD, Dance DA, et al.: Isolation of Pseudomonas pseudomallei from soil in north-eastern Thailand. Trans R Soc Trop Med Hyg. 1995; 89(1): 41–3. PubMed Abstract | Publisher Full Text
30. Göhler A, Trung TT, Hopf V, et al.: Multitarget Quantitative PCR Improves Detection and Predicts Cultivability of the Pathogen Burkholderia pseudomallei.
32. Limmathurotsakul D, Wuthiekanun V, Amornchai P, et al.: Effectiveness of a simplified method for isolation of Burkholderia pseudomallei from soil. Appl Environ Microbiol. 2012; 78(3): 876–7. PubMed Abstract | Publisher Full Text | Free Full Text

33. Limmathurotsakul D, Dance DA, Wuthiekanun V, et al.: Systematic review and consensus guidelines for environmental sampling of Burkholderia pseudomallei. PLoS Negl Trop Dis. 2013; 7(3): e2105. PubMed Abstract | Publisher Full Text | Free Full Text

34. Manivanh L, Pierret A, Rattanavong S, et al.: Burkholderia pseudomallei in a lowland rice paddy: seasonal changes and influence of soil depth and physico-chemical properties. Sci Rep. 2017; 7(1): 3031. PubMed Abstract | Publisher Full Text | Free Full Text

35. Interstate Technology & Regulatory Council Council: Analytical splitting and subsampling techniques. Incremental Sampling Methodology ISM-1. Technical and Regulatory Guidance. Washington, D.C: Interstate Technology & Regulatory Council, Incremental Sampling Methodology Team; 2012; 417.

36. Novak RT, Glass MB, Gee JE, et al.: Development and evaluation of a real-time PCR assay targeting the type III secretion system of Burkholderia pseudomallei. J Clin Microbiol. 2006; 44(1): 85–90. PubMed Abstract | Publisher Full Text | Free Full Text

37. Baker AL, Warner JM: Burkholderia pseudomallei is frequently detected in groundwater that discharges to major watercourses in northern Australia. Folia Microbiol (Praha). 2016; 61(4): 301–5. PubMed Abstract | Publisher Full Text

38. Dance DAB: Evaluation of consensus method for detection of B. pseudomallei in soil. 2018. http://www.doi.org/10.17605/OSF.IO/35GHQ
Open Peer Review

Current Referee Status: ✔ ✔

Version 2

Referee Report 05 December 2018

https://doi.org/10.21956/wellcomeopenres.16288.r34299

Rasana W Sermswan 1,2
1 Melioidosis Research Center, Faculty of Medicine, Khon Kaen University (KKU), Khon Kaen, Thailand
2 Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand

No further comment is required. The author responses fulfilled my requirement.

Competing Interests: No competing interests were disclosed.

Referee Expertise: Microbiology, Molecular biology.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Referee Report 07 November 2018

https://doi.org/10.21956/wellcomeopenres.16180.r34075

? Rasana W Sermswan 1,2
1 Melioidosis Research Center, Faculty of Medicine, Khon Kaen University (KKU), Khon Kaen, Thailand
2 Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand

The manuscript compared the performance of the consensus soil method with other methods to determine the distribution of B. pseudomallei in the soil. The authors selected Ban Nabone, Vientiane Province, Laos, some 560 km away from Ubon Ratchathani in Thailand where the consensus method was originally evaluated and found to have high sensitivity as their study area. This area was found to be much less sensitive by the consensus method than the conventional culture method. The authors concluded that no single method is adequate to conclude any environment to have or not have B. pseudomallei. The conventional culture method should be applied in a new environment.

My comments are as follows:

1. The samples were collected at two depths (30 cm and 60 cm) at 50 points to the total of 100 samples.
Even though the aim of this manuscript is to compare methods of detection of B. pseudomallei in the environment, however, results and discussions of other points of the study should be beneficial and worth to describe. What are the results from the different depth? Are they similar by all methods or different? Does the bacterium found equally in 2 depths?

2. Why was the consensus method that was applied in the same area with high sensitivity in the previous report found to be much less sensitive than conventional culture method in this study?

3. The authors discussed that the difference between the conventional culture method and the consensus method could not be explained by variations in culture media or in the experience of the staff reading the culture plates, as they controlled for all of these factors.

Could it be the soil type that makes them different? Conventional culture method mixed 100g of soil with 100ml water and incubated overnight before spreading on Ashdown medium while consensus method mixed the soil with water and the spread. Conventional culture, therefore, may be better for clay soil that holds bacterium stronger than sandy soil. Analysis of the soil types in this study may provide some clue. This may also help predicted suitable method for environmental study in new areas.

4. All methods used a different amount of soil. A hundred grams of soil used in conventional should be better than 10 gram. Does 100g or 10g used in conventional culture give similar sensitivity? The authors also mention that DNA extracted from 0.5g soil and used for PCR detection is not sensitive. However, 84 positives by 20g soil with enrichment method may be too high? How could the author make sure that there was no contamination or carryover of positive soil to the negative soil during DNA extraction or high sensitivity PCR amplification?

Minor comment:

Results and discussion

"There were, however, two samples in which B. pseudomallei was not detected by conventional culture but in which it was isolated using one of the consensus methods (in one case only in MORU and in the other in all three variations)."

This sentence may need be rewritten to make it more understandable.

Is the work clearly and accurately presented and does it cite the current literature? Yes

Is the study design appropriate and is the work technically sound? Partly

Are sufficient details of methods and analysis provided to allow replication by others? Yes

If applicable, is the statistical analysis and its interpretation appropriate? Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** Microbiology, Molecular biology.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

---

**Author Response 19 Nov 2018**

**David A. B. Dance,** Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit, Lao People's Democratic Republic

*We are grateful to the referees for taking the time to read and comment on our manuscript, which has enabled us to make improvements in Version 2. Our responses are given in italics below.*

**Referee 2**

The manuscript compared the performance of the consensus soil method with other methods to determine the distribution of B. pseudomallei in the soil. The authors selected Ban Nabone, Vientiane Province, Laos, some 560 km away from Ubon Ratchathani in Thailand where the consensus method was originally evaluated and found to have high sensitivity as their study area. This area was found to be much less sensitive by the consensus method than the conventional culture method. The authors concluded that no single method is adequate to conclude any environment to have or not have B. pseudomallei. The conventional culture method should be applied in a new environment.

My comments are as follows:

1. The samples were collected at two depths (30 cm and 60 cm) at 50 points to the total of 100 samples.

   Even though the aim of this manuscript is to compare methods of detection of B. pseudomallei in the environment, however, results and discussions of other points of the study should be beneficial and worth to describe. What are the results from the different depth? Are they similar by all methods or different? Does the bacterium found equally in 2 depths?

   *As the referee says, the purpose of this study was to compare the yield of the different methods and we feel that to include a discussion of this aspect would distract attention from the key message. For further details about the yield from different soil depths, readers are referred to Manivanh L, Pierret A, Rattanavong S, Kounnavongs O, Buisson Y, Elliott I, et al. Burkholderia pseudomallei in a lowland rice paddy: seasonal changes and influence of soil depth and physico-chemical properties. Scientific Reports. 2017;7(1):3031, and to the supplementary data of*...*
2. Why was the consensus method that was applied in the same area with high sensitivity in the previous report found to be much less sensitive than conventional culture method in this study?

The reason for the lower yield of the consensus method in Ban Nabone is not known at this stage, but some possible explanations are explored in paragraph 4 of the ‘Results and discussion’ section.

3. The authors discussed that the difference between the conventional culture method and the consensus method could not be explained by variations in culture media or in the experience of the staff reading the culture plates, as they controlled for all of these factors.

Could it be the soil type that makes them different? Conventional culture method mixed 100g of soil with 100ml water and incubated overnight before spreading on Ashdown medium while consensus method mixed the soil with water and the spread. Conventional culture, therefore, may be better for clay soil that holds bacterium stronger than sandy soil. Analysis of the soil types in this study may provide some clue. This may also help predicted suitable method for environmental study in new areas.

We agree that this is another possibility and have added the phrase “difference in the soil type” to the section referred to above.

4. All methods used a different amount of soil. A hundred grams of soil used in conventional should be better than 10 gram. Does 100g or 10g used in conventional culture give similar sensitivity? The authors also mention that DNA extracted from 0.5g soil and used for PCR detection is not sensitive. However, 84 positives by 20g soil with enrichment method may be too high? How could the author make sure that there was no contamination or carryover of positive soil to the negative soil during DNA extraction or high sensitivity PCR amplification?

Again, we agree that the different amounts of soil processed in the different methods could be an important factor and have added “and the amounts of soil processed in the different methods” into the section referred to above. We accept that contamination is always a potential problem with PCR methods, and we used a standard approach to minimise the risk of this by using physically separated rooms for DNA extraction, PCR preparation and DNA addition. Furthermore, no template controls were always negative. In addition, this would not account for the differences between the results obtained with DS/qPCR and ES/qPCR.

Minor comment:

Results and discussion

“There were, however, two samples in which B. pseudomallei was not detected by conventional culture but in which it was isolated using one of the consensus methods (in one case only in MORU and in the other in all three variations).”

This sentence may need be rewritten to make it more understandable.
We realise that this sentence is complex but we believe that it clearly describes the results obtained.

Competing Interests: No competing interests were disclosed.

---

Referee Report 25 October 2018

https://doi.org/10.21956/wellcomeopenres.16180.r34076

Ya-Lei Chen
Department of Biotechnology, National Kaohsiung Normal University, Kaohsiung, Taiwan

This manuscript is valuable to remind us that the isolation strategy for *B. pseudomallei* in the environment should strictly consider its own physiochemical parameters. Consensus methodology has shown successful isolation of *B. pseudomallei* from Thai soil but, in this report, it shows a worse isolation from Lao soil. I believe that *B. pseudomallei* strains exhibit many genomovars, morphovars and even pathovars in different areas that are endemic to melioidosis because *B. pseudomallei* accommodate in different soil condition through a long-term evolution by geographical seclusion. Thus, different isolation methods used in different places are reasonable. However, several concerns have to be considered:

1. Even if the soil was collected from same place, it was very difficult to ensure that presence or absence of *B. pseudomallei* or any organisms that were antagonistic to *B. pseudomallei* can be found in different pieces (sub-sample). Actually, the distribution of *B. pseudomallei* in soil sample is very uneven.

2. The suspect isolates were chosen following the colony morphology from consensus and ASH methods. However, this technique is experience-dependent because *B. pseudomallei* colony morphovars have been widely reported in these media. Several atypical morphologies such as smaller, moisturized or mucoid could appear in environmental isolates but they probably were ignored and seldom found in clinical isolates, usually appearing a dry and winkled colony morphology.

3. The fragment of *orf2* stretch of the TTSS could be amplified by soil organisms that are not related to *B. pseudomallei*. In other words, false positive probably existed in ES/qPCR method.

4. In Fig. 1, the proportion of positive sample in ES/qPCR is 84 or 85?

5. In the statistical analysis, there is no mention of how to calculate 95% CI.

Overall, I agree with the authors’ conclusion that *B. pseudomallei* in a new environment would be to use enrichment culture followed by PCR as a screening method. Repeatedly isolation for *B. pseudomallei* by selective media from PCR positive sample is needed.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes
Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

---

**Author Response 16 Nov 2018**

**David A. B. Dance,** Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit, Lao People's Democratic Republic

Evaluation of consensus method for the culture of *Burkholderia pseudomallei* in soil samples from Laos

**Response to Referees’ Comments**

*We are grateful to the referees for taking the time to read and comment on our manuscript, which has enabled us to make improvements in Version 2. Our responses are given in italics below.*

**Referee 1**

This manuscript is valuable to remind us that the isolation strategy for *B. pseudomallei* in the environment should strictly consider its own physiochemical parameters. Consensus methodology has shown successful isolation of *B. pseudomallei* from Thai soil but, in this report, it shows a worse isolation from Lao soil. I believe that *B. pseudomallei* strains exhibit many genomovars, morphovars and even pathovars in different areas that are endemic to melioidosis because *B. pseudomallei* accommodate in different soil condition through a long-term evolution by geographical seclusion. Thus, different isolation methods used in different places are reasonable. However, several concerns have to be considered:

1. Even if the soil was collected from same place, it was very difficult to ensure that presence or absence of *B. pseudomallei* or any organisms that were antagonistic to *B. pseudomallei* can be found in different pieces (sub-sample). Actually, the distribution of *B. pseudomallei* in soil sample is very uneven.

*We agree with the referee about this, which is why we went to considerable lengths to ensure that the sub-samples were as representative as possible by using the ‘2D Japanese slab-cake’ method, as mentioned in the ‘Methods’ section. We have added a paragraph in the ‘Results and discussion’ section to address this and other limitations of the study raised by the referees.*
2. The suspect isolates were chosen following the colony morphology from consensus and ASH methods. However, this technique is experience-dependent because *B. pseudomallei* colony morphovars have been widely reported in these media. Several atypical morphologies such as smaller, moisturized or mucoid could appear in environmental isolates but they probably were ignored and seldom found in clinical isolates, usually appearing a dry and winkled colony morphology.

*Again, we agree about this and have included this in the discussion of the limitations referred to above.*

3. The fragment of *orf2* stretch of the TTSS could be amplified by soil organisms that are not related to *B. pseudomallei*. In other words, false positive probably existed in ES/qPCR method.

*We are not clear of the referee’s evidence for this. This target has been widely used by others for the detection of *B. pseudomallei* in both clinical and environmental samples and is generally believed to be highly specific. However, since we accept that there are probably ‘near-neighbours’ of *B. pseudomallei* in the environment that have not yet been fully characterised, we have included this in the paragraph on limitations as above.*

4. In Fig. 1, the proportion of positive sample in ES/qPCR is 84 or 85?

*This was 84 of the 85 samples positive by any method as shown in the figure, the discrepancy being the result of the single sample that was negative by ES/qPCR but positive by conventional culture.*

5. In the statistical analysis, there is no mention of how to calculate 95% CI.

*We have added a statement in the ‘Statistical analysis’ section that says “The confidence intervals for sensitivities were estimated by using the ci command in STATA”.*

Overall, I agree with the authors’ conclusion that *B. pseudomallei* in a new environment would be to use enrichment culture followed by PCR as a screening method. Repeatedly isolation for *B. pseudomallei* by selective media from PCR positive sample is needed.

**Competing Interests:** None