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RESEARCH ARTICLE

REVISION **Evaluation of consensus method for the culture of *Burkholderia pseudomallei* in soil samples from Laos [version 2; referees: 2 approved]**

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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 Lao 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

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Referee Status:

	Invited Referees	
	1	2
version 2 published 21 Nov 2018		 report
version 1 published 15 Oct 2018	 report	 report

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- 2 **Rasana W Sermawan** , Khon Kaen University (KKU), Thailand
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Discuss this article

Comments (0)

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

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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; **Wuthiekanun V:** 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.

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REVISED 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 tropics¹. 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^{2–31}. 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 Thailand³², was published and proposed as a consensus methodology³³. 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 media³⁴. 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 published¹³, and this paper will focus on a comparison of the performance of the consensus soil method with other methods.

Methods

Sample collection

Soil sampling was performed during the dry season (April 2013) in a rice paddy near the village of Ban Nabone, Vientiane Province, Laos (18°22'51.4"N, 102°25'27.8"E, altitude 195 m), as previously described¹³. 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 culture³⁴ (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 method³⁵. 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.

i. 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

Table 1. Methods used to process each of 100 soil samples.

	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

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.* 2015¹³. 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. pseudomallei*^{2,36}. To reduce the effect of inhibitors, 400 ng/µl of bovine serum albumin (BSA, New England Biolab, USA) was added⁴.

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

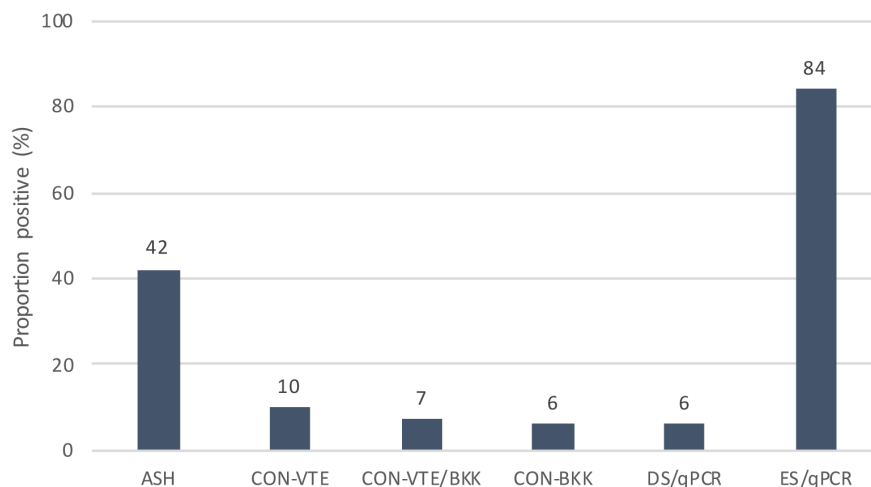


Figure 1. Proportion of samples that yielded *B. pseudomallei* by method. The number of the 100 samples in which *B. pseudomallei* was detected by each method is shown. Abbreviations: ASH, conventional semi-quantitative culture; CON-VTE, consensus method in LOMWRU using locally made media; CON-VTE/BKK, 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^{2,13,37}. 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 PCR³⁷. 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 environment³³. Although the consensus method has been successful in isolating *B. pseudomallei* from soil in many regions in Thailand¹⁰, 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>³⁸

Data are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

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Open Peer Review

Current Referee Status:  

Version 2

Referee Report 05 December 2018

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

 **Rasana W Serm Swan**  ^{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 Serm Swan**  ^{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, Kounnavongsa 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

this paper.

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 wrinkled 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 wrinkled 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