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Towards a rapid molecular diagnostic for melioidosis: comparison of DNA extraction methods from clinical specimens

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Abstract

Optimising DNA extraction from clinical samples for *Burkholderia pseudomallei* Type III secretion system real-time PCR in suspected melioidosis patients confirmed that urine and sputum are useful diagnostic samples. Direct testing on blood remains problematic; testing DNA extracted from plasma was superior to DNA from whole blood or buffy coat.

Burkholderia pseudomallei is a Gram-negative soil bacterium and the causative agent of melioidosis; a disease endemic in northern Australia and South East Asia (Cheng and Currie, 2005). Mortality from melioidosis can be most effectively decreased by early diagnosis and implementation of appropriate antibiotic therapy (Currie et al., 2010). Culturing *B. pseudomallei* from clinical samples is currently the gold standard for diagnosis of melioidosis; however definitive results can take up to seven days (Dance et al., 1989; Walsh et al., 1995). Molecular detection using a robust PCR target and sensitive DNA extraction methods can provide more rapid results but must overcome significant challenges when used on blood samples, including low bacterial numbers (Tiangpitayakorn et al., 1997) and the presence of PCR inhibitors (Al-Soud and Radstrom, 2001; Lau et al., 2010).

The Type III Secretion System (TTS-1) is a well-validated, species-specific PCR target for *B. pseudomallei* and forms the basis of an existing, widely-used real-time PCR assay (Meumann et al., 2006; Novak et al., 2006; Kaestli et al., 2007). However, there is no consensus on the best extraction method(s) for obtaining *B. pseudomallei* DNA from clinical specimens. In this study we used the existing TTS-1 real-time PCR assay, multiple types of clinical specimens obtained from melioidosis patients, and commercially available DNA extraction kits to determine the best extraction methods for detection of *B. pseudomallei* DNA in different clinical specimens.

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A total of 118 clinical specimens (48 blood, 41 urine and 29 sputum samples) were obtained from thirty patients enrolled at the Royal Darwin Hospital in tropical northern Australia with culture confirmed melioidosis. Ethics approval was granted by the Human Research Ethics Committee of the Northern Territory Department of Health and the Menzies School of Health Research (HREC 04/09), with written informed consent obtained from patients. Not all patients were able to provide enough specimens or blood volume to enable comparison of all extraction methods at once. For this reason, the following statistical analyses focused on comparing various pairings of extraction methods with DNA extracted from the same specimen. Moreover, although all specimens were from culture-confirmed melioidosis patients, it is not known if all specimens used in this study actually contained *B. pseudomallei* because they were collected separately and sometimes at different time points from the specimens that were culture positive.

DNA was extracted from clinical specimens using various extractions procedures as outlined in Table 1. Real-time PCR targeting the TTS-1 and performed on an ABI7900-HT machine (Life Technologies, Foster City, CA) was then used to confirm the presence/absence of *B. pseudomallei* DNA in each extract. Samples were tested in duplicate using 10 µL reactions with TaqMan Environmental Mastermix (Life Technologies), 0.9 µM primers and 0.25 µM of 5'-FAM labelled probe (Novak et al., 2006) (Biosearch Technologies, California, USA) with 4 µL of DNA. The cycling parameters were as previously described (Bowers et al., 2010) with the exception that 50 cycles were used instead of 40. A positive/negative cycle threshold (C_t) cut-off of 40 was set. Extraction negative controls and no-template PCR negative controls were included in every run and runs with false positive controls were excluded and repeated where possible. Statistical analysis comparing PCR results for different blood sample extraction methods from the same specimen was done by McNemar testing for presence (+ve)/absence (−ve) data and by Wilcoxon signed-rank test for comparison of C_t values.

For blood specimens, *B. pseudomallei* extraction from plasma using QIAamp (Qiagen, Hilden, Germany) proved best with more positive samples when compared pairwise with (i) whole blood extractions of the same blood specimen using PureGene (Qiagen) (8/13 positives vs. 0/13; $\chi^2(1) = 8$, $p=0.005$, Wilcoxon $p=0.006$); (ii) whole blood extractions using QIAamp (Qiagen) (7/13 positives vs. 3/13; $\chi^2(1) = 4$, $p=0.046$, Wilcoxon $p=0.0095$); and (iii) buffy coat extractions using QIAamp (Qiagen) (12/24 positives vs. 6/24; $\chi^2(1) = 4.5$, $p=0.034$, Wilcoxon $p=0.148$ (latter not significant)).

The MolYsis Complete5 kit (Molzym, Bremen, Germany) for whole blood extractions was trialled on a smaller number of specimens. This kit degrades inhibitory human DNA before bacterial cell lysis. On testing the same whole blood samples, there was a suggestion that MolYsis performed better than the PureGene kit (6/15 positives vs. 1/15; $\chi^2(1) = 3.6$, $p=0.059$, Wilcoxon $p=0.062$). However MolYsis did not perform better than QIAamp DNA extraction from plasma tested on the same blood samples (4/11 positives vs. 7/11; $\chi^2(1) = 1.3$, $p=0.257$, Wilcoxon $p=0.166$, not significant). When an additional bacterial cell lysis step using lysozyme was added to the MolYsis protocol, 3/3 samples were positive for *B. pseudomallei*.

Sputum and urine samples were processed as in Table 1. Of 29 sputum samples from 13 patients, 22 (76 %) were positive. All seven negative samples were from patients either never sputum culture positive or in whom cultures had become negative before the date of the PCR negative result. Of a total of 41 urine samples from 25 patients, 19 (46 %) were positive by at least one of the three methods used. There was no statistically significant difference when comparing results from whole 200 µL urine and pelleted 200 µL urine on the same sample and volumes of urine > 5 mL did not provide a significantly higher

positivity rate (data not shown). The median C_t values of positive samples were substantially lower for both urine and sputum samples than for blood samples (Median C_t value (95% confidence interval) for real-time PCR positive samples: urine 31.1 (27.0–36.0), sputum 33.0 (31.6–36.5), blood 37.7 (37.0–38.1)), consistent with both a higher bacterial load and potentially less PCR inhibition in sputum and urine.

This study confirms our previous findings that sputum and urine are useful clinical samples for diagnostic PCR testing in patients with melioidosis and standard DNA extraction methods are robust for these samples (Meumann et al., 2006). In that earlier study we found 100% sensitivity for real-time PCR in comparison to culture for sputum (14/14) and urine (5/5) samples. Furthermore it was not unusual for sputum and urine to be PCR positive in specimens from melioidosis patients which were culture negative. However that study also documented the sensitivity of PCR on blood samples from bacteremic melioidosis patients to be up to only 56% overall, depending on the sampling protocol (Meumann et al., 2006). A blood PCR positive rate of 74% was found for bacteremic melioidosis with septic shock.

Although molecular detection of *B. pseudomallei* in blood remains challenging, our findings support the use of plasma as the blood fraction with the highest proportional *B. pseudomallei* DNA recovery. The superior performance of *B. pseudomallei* DNA extraction from plasma over whole blood and buffy coat is consistent with previous reports of DNA extractions from different blood fractions for some other bacteria and fungi (Lau et al., 2010; Bourhy et al., 2011). Detection of circulating bacterial DNA (Bossola et al., 2009) or a lower amount of PCR inhibitors in plasma (Al-Soud and Radstrom, 2001) might contribute to this finding.

In conclusion, plasma is the preferred blood sample for diagnosis of melioidosis using PCR, although results with current methodology may still be less sensitive than traditional blood cultures. Additional steps that degrade inhibitory human DNA and facilitate bacterial cell lysis may improve the sensitivity of PCR on whole blood. PCR on sputum and urine specimens, however, provide results at least as good as traditional culture methods.

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Table 1

DNA extraction protocols

Specimen Type	Fraction	Kit	Starting Volume	Elution Volume	References / modifications
EDTA Whole Blood	N/A	MolYsis Complete5 (Molzym)	5 mL	100 µL	As per manufacturer's instructions except see footnote 1
EDTA Whole Blood	N/A	PureGene Blood Core Kit B (Qiagen)	5–10 mL*	250 µL	As per manufacturer's instructions.
EDTA Whole Blood	N/A	QIAamp DNA mini kit (Qiagen)	200 µL	100 µL	As per manufacturer's instructions.
EDTA Whole Blood	Plasma	QIAamp DNA mini kit (Qiagen)	5–10 mL whole blood (~3 mL plasma)	100 µL	As per manufacturer's instructions except see footnote 2
EDTA Whole Blood	Buffy coat	QIAamp DNA mini kit (Qiagen)	5–10 mL whole blood (~500 µL buffy coat)	100 µL	As per manufacturer's instructions.
Sputum	N/A	QIAamp DNA mini kit (Qiagen)	1–5 mL	100 µL	As per manufacturer's instructions except see footnote 3
Urine	Pellet	QIAamp DNA mini kit (Qiagen)	Up to 10 mL	100 µL	As per manufacturer's instructions except see footnote 2
Urine	Whole / Pellet	QIAamp DNA mini kit (Qiagen)	200 µL	100 µL	As per manufacturer's instructions.

Footnotes:

- 1) Suggested additional lysis step (see text): 100 µL of enzymatic lysis buffer (10 mg/mL lysozyme; 20 mM Tris-HCl, pH8.0; 2 mM EDTA; 1.2% Triton). Incubation at 37°C for 30 min (with *BugLysis* solution step). Final elution in AE buffer (Qiagen).
- 2) Plasma or 10 mL of urine were spun for 10 min at 4000 rpm and the pellet was used.
- 3) Sputolysin pretreatment (Calbiochem, Darmstadt, Germany) as per manufacturer's instructions. 5 × volume dilution of resulting pellet in 1 × PBS (Sigma-Aldrich, Sydney, Australia) of which 400 µL was used for the DNA extraction.

* Volumes of up to 10 mL of whole blood did not improve sensitivity in blood.