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Evaluation of a rapid diagnostic test for the detection of *Burkholderia pseudomallei* in the Lao People’s Democratic Republic

Running title

Melioidosis diagnosis by rapid antigen detection Laos

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Burkholderia pseudomallei causes significant global morbidity and mortality, with the highest disease burden in parts of Asia where culture-based diagnosis is often not available. We prospectively evaluated the Active Melioidosis Detect (AMD, InBios International, USA) lateral flow immunoassay (LFI) for rapid detection of B. pseudomallei in turbid blood cultures, pus, sputum, sterile fluid, urine, and sera. Performance was compared to B. pseudomallei detection using monoclonal antibody latex agglutination (LA) and immunofluorescence assays (IFA), with culture as the gold standard. AMD was 99% (99/100; 94.6 – 100%) sensitive and 100% (308/308; 98.8-100%) specific on turbid blood culture bottles, with no difference to LA or IFA. AMD specificity was 100% on pus (122/122; 97.0-100%), sputum (20/20; 83.2-100%), and sterile fluid (44/44; 92 – 100%). Sensitivity on these samples was: pus 47.1% (8/17; 23.0 – 72.2%), sputum 33.3% (1/3; 0.84 – 90.6%), and sterile fluid 0% (0/2; 0 – 84.2%). Urine AMD had a positive predictive value of 94% (32/34; 79.7 – 98.5%) for diagnosing melioidosis in our cohort. AMD sensitivity on stored sera, collected prospectively from melioidosis cases during this study, was 13.9% (5/36; 4.7% - 29.5%) when compared to blood culture samples taken on the same day. In conclusion, the AMD is an excellent tool for rapid diagnosis of melioidosis from turbid blood cultures, and maintains specificity across all sample types. It is a promising tool for urinary antigen detection, which could revolutionise diagnosis of melioidosis in resource-limited settings. Further work is required to improve sensitivity on non-blood culture samples.
Introduction

Burkholderia pseudomallei is a saprophytic bacterium causing melioidosis, a disease with diverse clinical manifestations including fulminant septicaemia, pneumonia, meningo-encephalitis, abscess formation, septic arthritis and more indolent cutaneous presentations (1). The global burden of melioidosis has been estimated at 165,000 cases/year with 89,000 fatalities (2). The highest known burden of disease is in south and southeast Asia where case fatality rates reach around 40% (3), and even in countries with advanced healthcare systems such as Australia, mortality reaches 14% (4). In the Lao People’s Democratic Republic (Laos), increasing numbers of cases have been recognized since the disease was first diagnosed in 1999 (5), and more than 100 cases of culture-confirmed melioidosis are now being detected at Mahosot Hospital Microbiology Laboratory each year with peak incidence during the rainy season (6).

Culture is currently the gold standard diagnostic test for melioidosis with 100% specificity but an estimated sensitivity of only 60% (7). However, culture confirmation takes a minimum of 48-72hrs (7), requires specific media for optimal sensitivity in non-sterile samples (8), and laboratory containment and expertise often not available in endemic areas. B. pseudomallei is intrinsically resistant to many antibiotics and melioidosis therefore does not respond to most agents used for empiric treatment of sepsis, pneumonia and abscesses in developing countries (9). Therefore, life-saving treatment is often fatally delayed if a specific diagnosis cannot be confirmed. Simple, rapid diagnostic tests for melioidosis for use directly on clinical samples are needed, not only
for improving patient outcomes but also to improve epidemiological
surveillance of melioidosis and thereby strengthen public health interventions.
Immunofluorescence to detect *B. pseudomallei* directly in clinical samples
(10) and latex agglutination for rapid identification of positive cultures (11, 12)
are used in some melioidosis-endemic areas. However, these require
equipment, expertise and reagents that are not widely available. More
recently, an immunochromatographic lateral flow rapid diagnostic test (RDT)
to detect *B. pseudomallei* extracellular polysaccharide antigen directly in
clinical samples has been developed (Active Melioidosis Detect (AMD),
InBios, USA) (13). The method is simple, rapid (result within 15 minutes),
relatively cheap (estimated $2/test), does not require additional equipment,
and the kit may be stored at room temperature, making it ideal for use in
resource-limited settings. To date this kit has undergone only limited clinical
evaluations (13, 14). We evaluated the diagnostic performance of AMD on a
variety of clinical samples (including blood culture broths, pus, sterile fluid,
sputum, and urine) over two rainy seasons from patients with suspected and
culture-confirmed melioidosis presenting to Mahosot Hospital, Vientiane,
Laos.

Results

Analytical sensitivity and specificity

All *B. pseudomallei* seeded blood cultures were AMD-positive by 12hrs
incubation (Table S1). The lower limit of detection of the AMD was found to be
1.4 x 10^5 CFU/ml, meaning that it was positive in 3 of 4 seeded blood cultures
before they became visibly turbid and in 4/4 before organisms were
detectable by Gram staining of the broth. Centrifugation of blood culture broth prior to performing AMD did not reduce time to positivity.

All non-\textit{B. pseudomallei} reference strains were negative by AMD (Table S2). Two soil isolates of \textit{B. thailandensis} and one sputum isolate of \textit{B. cepacia} were AMD-positive. All 3 of these isolates were known to give a positive \textit{B. pseudomallei} latex agglutination test, probably due to production of a cross-reacting extracellular polysaccharide (15). All four positive control \textit{B. pseudomallei} clinical isolates were positive by AMD.

\textbf{Prospective evaluation}

Between 26\textsuperscript{th} June and 18\textsuperscript{th} December 2014, 89 patients were diagnosed with melioidosis by culture of \textit{B. pseudomallei} from at least one sample type (blood, pus, sputum, sterile fluid, urine, throat swab, wound swab (Figure S1)). Median age was 45 years (IQR 27-54 years), 55\% were male and 20/89 (22.5\%) patients died during admission or were discharged moribund. Blood cultures were received from 85 of these patients (85/89, 96\%) of whom 54\% (46/85) were bacteraemic with \textit{B. pseudomallei}. Of the 43 patients not found to be bacteraemic, 34 had localized disease whilst 9 had evidence of multifocal disease.

\textit{Turbid blood cultures}

There were 412 turbid blood culture bottles during the study period, of which 4 did not have AMD performed and were therefore excluded. 408 turbid blood culture bottles from 247 patients were thus included in the analysis.
Organisms isolated from the 408 blood cultures are shown in Figure S2. B. pseudomallei was isolated from 100 bottles (Figure 1a). Overall AMD sensitivity was 99% (99/100; 94.6 – 100%) and specificity 100% (308/308; 98.8-100%). 252 of these 408 turbid blood culture bottles had Gram-negative bacilli (GNB) seen on Gram stain and therefore had additional rapid tests performed: latex agglutination (n=237), immunofluorescence assay (IFA; n=176) (Figure 1a). Sensitivities and specificities for these tests are given in Table 1. 166 turbid blood culture samples with GNB seen on microscopy had all 3 rapid tests performed, with agreement between all 3 rapid tests and culture in 98.2% (163/166). 1 sample was AMD and latex agglutination negative but IFA and B. pseudomallei culture positive. 2 samples were scanty positive by IFA (1-3 bacilli/100 fields) but negative by all other tests.

**Pus**
139/150 pus samples received during the study period had AMD performed and were included in the analysis. B. pseudomallei was cultured from 17/139 samples. 95/139 pus samples also underwent IFA (Figure 1b). AMD was significantly more specific than IFA (p=0.0006; Table 1). However, all 8 false positive IFA results were reported as scanty (1-5 bacilli/100 fields).

**Sputum**
26 sputa were received during the study period, 3 of which did not have AMD performed and were excluded. The remaining 23 sputa underwent both AMD and IFA testing and no significant difference in sensitivity or specificity was found (Table 1). 3/23 samples were B. pseudomallei culture positive, all 3 of
which were IFA positive but only 1 of which was AMD positive (Figure 1c).

There were 3 positive IFA tests from culture negative samples, but all were reported as scanty (1-7 bacilli/100 fields), and 2 of these samples were from the same patient who, although culture negative for B. pseudomallei, had a clinical picture compatible with melioidosis and died shortly after transfer to Thailand for further healthcare.

Sterile Fluid

Between 10\textsuperscript{th} October and 18\textsuperscript{th} December 2014 50 sterile fluid samples were received, 46 underwent AMD and were included in the analysis (29 pleural fluid; 6 joint fluid; 2 pericardial fluid; 9 ascitic fluid). 42/46 also underwent IFA. B. pseudomallei was isolated from 2 samples (both joint fluid from the same patient, samples taken 11 days apart); IFA was positive on both these samples whilst AMD was negative on both (Figure 1d). The sensitivity of IFA was therefore significantly better than AMD (Table 1) although numbers are small, and confidence intervals wide.

Urine

Between 2\textsuperscript{nd} July and 2\textsuperscript{nd} Sept. 2014 249 urine samples were received, AMD was not performed on 28 and 16 were duplicate specimens, thus 205 were included in the analysis (Figure 1e.i). 3/205 urine samples were B. pseudomallei culture positive, 2 of which were AMD positive (sensitivity 66.7% (9.4 - 99.2%)). The B. pseudomallei culture positive urine sample which was AMD negative had a low bacterial load with only 1 colony forming unit isolated from a centrifuged deposit. AMD specificity was 100% (98.2 - 100%);
Organisms isolated in culture from all 205 urines are shown in Figure S3.

From 3rd September onwards only selected urines were included in the study (3rd Sept. - 18th December 2014 n=102; 23rd June – 12th Nov. 2015 n=189).

AMD was not performed on 27 samples (2014: 5; 2015: 22 samples), and 23 samples were duplicates and therefore excluded. Thus 241 samples were included in the analysis. 15/241 urine samples were *B. pseudomallei* culture positive, 13/15 of which were AMD positive (Table 1). 21/226 urine culture negative samples were AMD positive (Figure 1e.ii). Interestingly 19/21 of these urines came from patients who had melioidosis confirmed by culture from another site suggesting that these were not “false positive” AMD results but that the AMD was detecting true *B. pseudomallei* antigenuria. The positive predictive value of AMD on urine for correctly diagnosing melioidosis in this cohort was therefore 94.1% (32/34; 79.7 – 98.5%) with a disease prevalence of 35.7% (86/241).

*Urine samples from melioidosis cases.* To further describe *B. pseudomallei* antigenuria in our cohort results of urine samples received from the 182 culture-confirmed (from any site) melioidosis cases during the 2014 and 2015 study periods were analyzed further. A urine sample was received from 114 (2014: 57; 2015: 57) of these 182 patients, 20/114 urines were culture positive for *B. pseudomallei* (16/20 were AMD positive). 21 of the 94 culture-negative urines were AMD positive (Table 2). Patients who were urine culture negative for *B. pseudomallei* but had disseminated melioidosis were significantly more likely to be urine AMD positive than those with localized melioidosis (18/61 vs 3/32; p = 0.036). Presence of *B. pseudomallei* bacteraemia did not increase
the likelihood of urine AMD positivity overall (15/49 vs 6/44, p = 0.08), or in patients with disseminated melioidosis (15/49 vs 3/12, p = 1.0).

Urine concentration. Due to lack of availability of urine concentrators this was performed retrospectively on 20 stored urine isolates collected prospectively between 2nd July and 18th December 2014. All urine samples were from confirmed melioidosis cases but were AMD negative on neat urine and B. pseudomallei urine culture negative. 6 of 20 urine samples were AMD positive after urine concentration.

Sera
71 stored serum samples from the 89 melioidosis cases diagnosed from 26th June – 18th December 2014 were available for AMD testing (Figure 1f). 5/71 (7%) samples were AMD positive. Each of these five patients were culture-positive for B. pseudomallei from blood taken on the same day as the serum sample. Of the patients negative by AMD on sera, 31/66 had been bacteraemic with B. pseudomallei on the same day as the serum sample was taken, and a further 9 non-bacteraemic patients had evidence of disseminated disease. Sera therefore had low sensitivity for diagnosis of melioidosis when compared with blood culture as the gold standard, 13.9% (5/36; 4.7% - 29.5%).

Discussion
We evaluated accuracy of the AMD lateral flow immunoassay (LFI) for the rapid diagnosis of melioidosis directly from clinical samples. The LFI detects
extracellular polysaccharide of \textit{B. pseudomallei} with a limit of detection (LOD) of 0.2ng/mL (13, 16). On turbid blood cultures the AMD was found to have excellent analytical and diagnostic sensitivity and specificity, comparable to both IFA and latex agglutination which were found to have similar performance characteristics to previous studies (11, 17-19). One turbid blood culture bottle was AMD and latex agglutination negative but culture positive for \textit{B. pseudomallei}. After 24 hours further incubation both tests were positive on the same bottle suggesting that the initial false negative results may reflect an initial bacterial load lower than the limit of detection for both tests. The high specificity of the AMD on turbid blood cultures is extremely promising for deployment to field settings in the tropics, where simple tests such as the AMD may be performed after a broth incubation step in the absence of laboratory facilities for Gram stain and culture. A positive AMD result, potentially obtained as early as 12 hours post blood sampling, could prove life-saving if antimicrobial treatment is adapted appropriately. The AMD maintained excellent analytical and diagnostic specificity across all sample types, even ‘non-sterile’ samples such as sputum and urine, which are more likely to contain contaminating bacteria (including environmental \textit{Burkholderia} spp. in an endemic tropical setting). False positive AMD results were only seen in our study with environmental \textit{Burkholderia} strains known to express a similar extracellular polysaccharide to that detected by the AMD. Although this may pose a problem when the AMD is used for detection of \textit{B. pseudomallei} in environmental samples such as soil (20), in clinical samples it is rarely likely to be relevant. The finding of AMD reactivity in a clinical strain of \textit{B. cepacia} in this study is novel, as previous testing of \textit{B. cepacia} complex
strains had not demonstrated this (unpublished observations, D. AuCoin).

Relatively few sputum samples were included in this study and further work is needed to investigate how frequently respiratory samples containing non-

pseudomallei Burkholderia (for example from patients with cystic fibrosis) may cause AMD reactivity. However, in our experience such strains are rare and the majority of B. cepacia isolates do not cross react in this way (data not shown).

AMD analytical sensitivity was extremely promising, with AMD LOD on broth cultures better than previous estimates of LOD for latex agglutination (1 – 2 x10^6 CFU/ml (18)), and similar to estimated bacterial loads of B.
pseudomallei in urine (1.5 x 10^4 CFU/ml), sputum (1.1 x 10^5 CFU/ml), and pus (1.1x10^7 CFU/ml) samples (21). However, diagnostic sensitivity was disappointing in non-blood culture samples. We observed that viscous samples were more challenging to process for AMD and this, coupled with low initial bacterial loads, may explain the moderate sensitivity observed in these samples. Seven pus samples in our study were received in broth from distant study sites (and therefore excluded from the main analysis) and AMD was positive in all 3 samples subsequently B. pseudomallei culture positive. An initial enrichment culture step has been previously suggested to improve the sensitivity of IFA for B. pseudomallei from non-blood clinical samples (10). It is likely that broth incubation of pus, sputum and sterile fluid found to be AMD negative on direct testing will increase AMD sensitivity on these samples, whilst only delaying diagnosis by a few hours, although appropriate laboratory bio-safety equipment and practices would be needed.
IFA diagnostic sensitivity and specificity in this study were similar to previous reports (10). However, despite the reportedly lower LOD (2×10^3 CFU/ml of IFA (10), IFA was not found to be significantly more sensitive than AMD on any sample type except sterile fluids, nor was IFA significantly more specific than AMD except on pus samples. The inherent subjectivity of immunofluorescent microscopy, the difficulty of misidentifying fluorescent debris as bacteria, and the labour and resource-intensive methodology are important disadvantages of IFA compared with AMD.

The number of B. pseudomallei culture-positive urine samples in this study was limited, however the ability of the AMD to detect B. pseudomallei antigenuria in melioidosis patients, particularly those with disseminated melioidosis whose urine is culture-negative for B. pseudomallei, is encouraging. Our findings replicate previous work in a non-human primate model, in which antigen was detectable in urine by AMD as early as 2-3 days after experimental infection with B. pseudomallei (D. AuCoin personal communication). The extracellular polysaccharide detected by the AMD maintains its molecular weight in urine without degradation over time which might otherwise affect the sensitivity of the AMD on urine (22). Urine is an easily available, non-invasive sample and a simple matrix for the AMD. Reliable urine antigen detection for melioidosis by a rapid test such as the AMD could revolutionize diagnostics in resource-limited parts of the world where this disease is most prevalent. However, the overall sensitivity of urine AMD for detection of melioidosis in our cohort was only 33% (37/114; 24 – 41.9%). Use of simple table-top urine concentrators increased AMD sensitivity in this study, however numbers tested were limited and further work is needed.
to establish the extent to which urine concentration improves AMD sensitivity in urine. At approximately $6/ sample, cost may prove a barrier to the use of this concentration technique for urine samples in endemic settings.

The AMD is an extremely promising tool for diagnosis of melioidosis worldwide and meets all ASSURED criteria for RDTs (23), although further work is required to optimize sensitivity on non-blood samples. However, it is not yet a true ‘Point of Care’ test, with AMD sensitivity on stored whole blood samples from bacteraemic melioidosis patients having previously been shown to be 40% (16/40; compared with 20% sensitivity for molecular detection on the same samples) (14), and on stored serum samples in this study only 7% (5/71). The low bacterial load in blood samples (1 CFU/ml prior to broth incubation (13)) may limit the utility of the AMD on these sample types and further large-scale prospective evaluations are required. However, the simple nature of the AMD technology means that it is more likely than molecular diagnostics to be widely applicable in a developing country context for the foreseeable future.

The main limitation of this study was that not all samples underwent testing by all the relevant rapid tests. This was partly related to the nature of the study with the assays being performed during routine processing in a busy diagnostic laboratory. However, this study design gave a realistic indication of assay performance in a routine setting. In addition, the immunofluorescence assay, which had not previously been routinely used in our laboratory, took longer than expected to optimize and therefore turbid blood cultures and pus received in the initial stages of the study did not undergo IFA. Sputum and sterile fluid analyses were also limited due to low sample numbers.
In some patients in our study a positive urine AMD result was the first indication of melioidosis, preceding culture confirmation by at least 24hrs and resulting in early switch to appropriate antibiotics in critically unwell patients. This study was not designed to evaluate the clinical impact of obtaining a rapid diagnosis of melioidosis by AMD, however now that we have shown diagnostic specificity to be excellent, we are further investigating this. Throat swabs are another easily obtained sample type that is routinely used for diagnosis of melioidosis by culture in our setting. Incubation of a throat swab in liquid selective enrichment broth such as SBCT (8) would enable throat swabs to be used for rapid diagnosis of melioidosis by AMD, and this also warrants further study.

In conclusion, the AMD has excellent sensitivity and specificity for early detection of *B. pseudomallei* in blood culture broth. It also has the advantage over the latex agglutination test that it can be used directly on other sample types for the diagnosis of melioidosis. Specificity is retained when used directly on these other samples, but sensitivity is only moderate and requires optimization. Diagnosis of melioidosis from urine using AMD may significantly enhance diagnosis of this neglected disease. Studies are needed in a variety of different prevalence settings in order to truly understand the utility of this assay, however deployment of the AMD globally could improve our understanding of the epidemiology of melioidosis. Ultimately, we hope to see LFI technology used to multiplex antigen detection for a number of important causes of febrile illness in resource-limited settings.
Materials and Methods

Analytical Sensitivity and Specificity

The limit of detection of the AMD was assessed using seeded blood cultures and quantitative culture techniques. A single colony of *B. pseudomallei* (clinical isolate) was taken from a 24-48 hour culture and a 0.5 McFarland suspension made using 5ml phosphate buffered saline (PBS). This was diluted to 1:1,000,000 and then 1 ml inoculated into each of 4 negative blood culture bottles (Pharmaceutical factory No. 2, Vientiane, Laos; (24)), shaken gently to mix and incubated aerobically at 35-37°C. From time of inoculation (Time 0) and 12 hourly thereafter blood culture broths were observed for turbidity, Gram stain was performed using standard methods, and AMD and quantitative culture were performed. AMD was performed in duplicate on paired uncentrifuged and centrifuged samples from each blood culture broth as follows: 0.5ml of blood culture broth mixture was dispensed into a 1.5ml Eppendorf tube, 1 drop of lysis buffer was added and the suspension gently mixed using a micropipette. 20µl of this suspension was then applied to the AMD test strip, followed by 3 drops of chase buffer. The result was read after 15 minutes according to manufacturers instructions (Appendix 1). For centrifuged samples 1ml of blood culture broth was centrifuged at 845g (3000rpm on Microcentrifuge 5424 Eppendorf) for 10 minutes, the pellet then re-suspended in 1 drop of lysis buffer and 20µl of this suspension applied to the AMD test strip and read as above. All centrifugation was carried out in Biosafety Containment Level 3.

Quantitative culture was performed using the Miles and Misra method (25): 1ml was removed from each of the seeded blood culture broths after 0, 12
and 24hrs of incubation. Each 1ml was diluted 1:10 in PBS 8 times to make dilutions of $10^{-1}$ to $10^{-8}$ concentration. 20µl of each of these dilutions was pipetted onto 1/6th of a blood agar plate, allowed to dry for 20-30 minutes, and then incubated aerobically at 35-37°C for 24hours. Each dilution was tested in triplicate. Colony-forming units/ml (CFU/ml) were calculated for each seeded blood culture as: average number of colonies for the dilution where the highest numbers of discrete colonies (between 10-100) were clearly seen x 50 x dilution factor. The result of quantitative culture was recorded and compared to AMD positivity across the panel of 4 seeded blood cultures to estimate AMD limit of detection.

To investigate analytical specificity, negative blood culture broths were seeded (as above) with a range of NCTC/ATCC reference organisms (Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC 700603, Klebsiella oxytoca NCTC 8167, Enterobacter aerogenes NCTC 10006, Enterobacter cloacae NCTC 11580, Citrobacter freundii NCTC 9750, Edwardsiella tarda NCTC 10396, Salmonella Typhi NCTC 786, Salmonella Enteritidis ATCC 13076, Pseudomonas aeruginosa ATCC 27853, Acinetobacter baumannii NCTC 12156, Ochrobactrum anthropic NCTC 12168, Aeromonas hydrophila NCTC 8049, Yersinia enterocolitica NCTC 11175, Vibrio cholera NCTC 8021, Burkholderia thailandensis NR-9908, Burkholderia cepacia NCTC 10743, Staphylococcus aureus ATCC 29213, Staphylococcus epidermidis NCTC 11047), soil isolates of Burkholderia thailandensis, and Lao clinical isolates of B. pseudomallei and B. cepacia (Table S2). Broths were incubated aerobically at 35-37°C and observed daily for turbidity. AMD was performed on uncentrifuged samples from turbid blood culture broths (as previously described).
Prospective Evaluation

Study population

Between 26th June and 18th December 2014 all turbid blood cultures, pus and sputum samples received at Mahosot Hospital Microbiology Laboratory, Vientiane, Laos were included. Sterile fluid samples received between 10th October and 18th December 2014 were also included. All routine urine samples received between 2nd July and 2nd September 2014 were included in order to provide baseline specificity data. After an interim analysis, selected urine samples only were included (3rd Sept – 18th December 2014 and 23rd June – 12 November 2015). Selection criteria were presence of ≥1 of the following clinical details: suspected or confirmed melioidosis; diabetes mellitus and sepsis or fever; prostatitis; lung, liver, or spleen abscess. Only the first urine sample received from a patient was included.

Stored admission sera from patients diagnosed with melioidosis by culture from any sample between 26th June and 18th December 2014 were retrospectively tested by AMD in June 2015.

Sample processing

All samples were processed for microscopy and culture according to standard laboratory procedures (CLSI). B. pseudomallei was isolated from clinical samples using standard (goat blood, chocolate and MacConkey agars), and specific selective media (Ashdown’s agar and modified Ashdown’s selective broth (8)). Each set of blood cultures sent from a patient at any one time contained two blood-broth culture bottles (Pharmaceutical factory No. 2,
Vientiane, Laos; (24)), which were incubated aerobically at 35-37°C and observed daily for turbidity for up to 7 days.

**Blood cultures:** AMD was performed on all turbid blood culture broths. Turbid blood cultures with Gram-negative bacilli seen on microscopy also underwent *B. pseudomallei* latex agglutination and immunofluorescence assay (IFA) testing. AMD method was: 0.5ml of turbid blood culture broth dispensed into a 1.5ml Eppendorf tube, 1 drop of lysis buffer added and the suspension gently mixed using a micropipette. 20µl of suspension was added to 3 drops of chase buffer in a 0.6ml Eppendorf tube, and mixed by gentle pipetting. The AMD test strip was then inserted and the result read after 15 minutes according to manufacturer’s instructions (Appendix 1). Latex agglutination was performed as previously described (17): 5µl of latex reagent was mixed with one drop of uncentrifuged blood culture broth on a glass slide, gently rocked and observed for agglutination within 2 minutes. Positive (heat killed *B. pseudomallei*) and negative (heat killed *B. thailandensis*) controls were performed each day by mixing 5µl control with 5µl of latex reagent.

IFA, based on the previously described method (19), was performed as follows: 10µl of turbid blood culture broth was spread on a glass slide to create a thin smear and allowed to air dry. The slide was then fixed by flooding with absolute methanol for 10 minutes at room temperature, and again allowed to air dry. 10µl of IFA reagent (containing 5µg/ml monoclonal antibody 4B11 and 20µg/ml Alexa Fluor® 488 conjugated-Goat Anti-Mouse IgG) was then applied to the smear, covered with a coverslip and incubated at room temperature for 5 minutes before observing under a fluorescent...
microscope (Nikon Eclipse E600 microscope with the U-FL Epifluorescence attachment) at a magnification of x1000 using oil immersion. Periphery of bacilli showing strong apple-green fluorescence was recorded as a positive result (10). Positive results were semi-quantified using a scheme adapted from the International Union against Tuberculosis and Lung Disease (IUATLD) guidelines for quantification of Acid Fast Bacilli (AFB) (26) (Table S3). A minimum of 100 fields were examined before slides were recorded as IFA negative (i.e. no apple-green fluorescent bacilli seen). IFA was performed on the same day that the smear was made whenever possible, and the next day if not. Slides of heat-killed (1 x 10^6 CFU/ml in PBS at 80°C for 1 hour) B. pseudomallei and B. thailandensis were used as positive and negative controls, respectively, for each batch of immunofluorescence tests. Routine laboratory staff performed the latex agglutination and AMD tests, whilst all IFA tests were performed by LB blinded to the latex and AMD test results. All blood culture work was performed in a Biosafety Cabinet at Laboratory containment level 2 according to normal local practice.

*Pus, sputum, sterile fluid:* AMD and IFA were performed on all pus, sputum, and sterile fluid samples on receipt in the laboratory. Latex agglutination is not validated for these sample types and was therefore not performed on these samples. IFA was performed as above using 1µl of sputum, pus or sterile fluid sample to make the original smear. AMD was performed as follows: 20µl of pus, sterile fluid or thick/viscous sputum samples was mixed with 3-4 drops of lysis buffer by vortexing for 15 seconds. 20µl of this mixture was then added to 3 drops of chase buffer in a 0.6ml Eppendorf tube and mixed by
gentle pipetting. An AMD test strip was inserted and the result read after 15 minutes. Thin/watery sputum samples were processed in the same way except that 50µl of sample was mixed with 2-3 drops of lysis buffer initially. Routine laboratory staff performed AMD tests, while IFA was performed by LB blinded to the results of AMD.

Urine: AMD was performed using 50µl of neat urine added to 3 drops of chase buffer in a 0.6ml Eppendorf tube and mixed by gentle pipetting. AMD test strip was then inserted and the result read after 15 minutes. AMD tests were performed by routine laboratory staff. If AMD on neat urine was negative then urine concentration was performed when concentrators (Minicon B15, Merck Millipore Ltd) were available. 5ml of urine was concentrated x100 using these simple table-top concentrators according to manufacturer’s instructions. AMD was then performed as above using 20µl of concentrated urine. Neither latex agglutination nor IFA have been validated for use directly with urine samples and were therefore not performed on these samples.

Sera: Admission sera (stored at -80°C) from culture-confirmed melioidosis cases were retrieved and AMD performed as follows: 35µl serum added to the AMD test strip followed by 3 drops of Chase buffer. Results were read after 15 minutes as previously.

Each new box of AMD test strips underwent quality assurance by testing one strip from the box as follows: a single colony of *B. pseudomallei* positive control (Lao clinical isolate UI 8976) was emulsified, using a sterile loop, in 2
drops of lysis buffer. 3 drops of chase buffer were then added to this bacterial suspension and mixed gently by pipetting before the AMD test strip was inserted and read as previously described.

Data Analysis

Diagnostic sensitivity and specificity of the AMD, latex agglutination and IFA on the different sample types were calculated using culture as the reference standard. The sensitivity and specificity of AMD was compared with latex agglutination and IFA on turbid blood cultures containing Gram-negative bacilli, and with IFA alone for pus, sputum, and sterile fluid samples using a two-sample test of proportions. In patients confirmed with melioidosis (by culture from any sample) whose urine was culture negative for *B. pseudomallei* a two-sided Fisher's exact test was used to analyze the association between urine AMD positivity and 1) disseminated vs. localized melioidosis, and 2) presence or absence of *B. pseudomallei* bacteraemia. Melioidosis cases were defined as ‘disseminated’ when *B. pseudomallei* bacteraemia was present and/or there was clinical/ radiological or microbiological evidence of multiple sites of disease. Cases were defined as ‘localized’ if only one site of disease was present e.g. pneumonia or parotitis. Analysis was done using STATA, v14.2 (College Station, TX, USA).
Acknowledgements

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Author contributions: DD and DA conceived of and designed the study; CNF performed the analytical sensitivity and specificity work; KW, LB, CNF and VD developed the methodology, processed and tested samples; KW and CNF collated and analyzed the data; SL performed the statistical analysis; KW prepared the manuscript; all authors reviewed and revised the final manuscript.

Conflict of interests: DA declares that InBios has licensed the mAb 4C4, produced by the AuCoin laboratory, from the University of Nevada, Reno.

There are no other conflicts of interest to declare.
Appendix 1. Interpretation of AMD reactivity according to manufacturer’s (InBios) instructions:

C = control line; T = test line. Note a faint test line is considered positive as the red colour in this region varies depending on the concentration of antigen present. Examples of positive AMD results are given below.
References


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Figure Legends

Figure 1. Sample Flow Diagrams ("not documented" = inadequate documentation to include result in analysis; "missed" = test not performed)

Figure 1a. Sample Flow diagram: Turbid blood cultures with Gram-negative bacilli seen on microscopy

Figure 1b. Sample Flow diagram: Pus samples

Figure 1c. Sample Flow diagram: Sputa samples

Figure 1d. Sample Flow diagram: Sterile Fluid samples

Figure 1e. Sample Flow diagram: Urine samples

i. Urine samples - Unselected (2nd July – 2nd September 2014)

ii. Urine samples – Selected (3rd Sept – 18th Dec 2014; 23rd June – 12 Nov 2015)

Figure 1f. Sample Flow diagram: Serum samples from culture-confirmed melioidosis cases diagnosed 26th June – 18th December 2014
Table 1. Diagnostic sensitivity and specificity for all tests and sample types compared with culture as the reference standard.

<table>
<thead>
<tr>
<th>Specimen type</th>
<th>Test</th>
<th>Sensitivity % (95% Confidence Interval)</th>
<th>Specificity % (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turbid Blood culture with GNB (252)</td>
<td>AMD (252)</td>
<td>99.0 (94.6-100%)</td>
<td>100 (97.6-100%)</td>
</tr>
<tr>
<td></td>
<td>Latex (237)</td>
<td>99.0 (94.5 – 100)</td>
<td>100 (97.4 – 100)</td>
</tr>
<tr>
<td></td>
<td>IFA (176)</td>
<td>100 (94.8 – 100)</td>
<td>98.1 (93.4 – 99.8)</td>
</tr>
<tr>
<td>Pus (139)</td>
<td>AMD (139)</td>
<td>47.1 (23.0 - 72.2)</td>
<td>100 (97.0-100)</td>
</tr>
<tr>
<td></td>
<td>IFA (95)</td>
<td>66.7 (29.9 – 92.5)</td>
<td>90.7 (82.5-95.9)</td>
</tr>
<tr>
<td>Sputum (23)</td>
<td>AMD (23)</td>
<td>33.3 (0.84 – 90.6)</td>
<td>100 (83.2-100)</td>
</tr>
<tr>
<td></td>
<td>IFA (23)</td>
<td>100 (29.2 – 100)</td>
<td>85.0 (62.1-96.8)</td>
</tr>
<tr>
<td>Sterile Fluid (46)</td>
<td>AMD (46)</td>
<td>0 (0 – 84.2%)</td>
<td>100 (92.0 – 100)</td>
</tr>
<tr>
<td></td>
<td>IFA (43)</td>
<td>100 (15.8 – 100)</td>
<td>100 (91.4-100)</td>
</tr>
<tr>
<td>Urine</td>
<td>p-value</td>
<td>0.046</td>
<td>1.0</td>
</tr>
<tr>
<td>-------</td>
<td>---------</td>
<td>-------</td>
<td>-----</td>
</tr>
<tr>
<td>Selected</td>
<td>AMD (241)</td>
<td>86.7 (59.5 – 98.3%)</td>
<td>90.7 (86.2-94.2%)</td>
</tr>
</tbody>
</table>

NS = not significant; * p = 0.994 for AMD v latex, 0.402 for Latex v IFA, 0.405 for IFA v AMD; ** p = 1.0 for AMD v latex, 0.107 for Latex v IFA, 0.169 for IFA v AMD.
Table 2. Urine AMD results according to site of disease in melioidosis cases who were urine culture-negative for *B. pseudomallei* (n = 93*).

<table>
<thead>
<tr>
<th>Site of disease</th>
<th>Urine AMD result</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Disseminated (61)</td>
<td>Bacteraemic (49)</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Not bacteraemic (12)</td>
<td>3</td>
</tr>
<tr>
<td>Localized (32)</td>
<td></td>
<td>3</td>
</tr>
</tbody>
</table>

*1 patient did not have sufficient data available to categories site of disease.
Sputum samples received (n=26)

AMD not performed (n=3)
  - *B. pseudomallei* isolated (n=0)

Sputum samples included (n=23)

AMD performed (n=23)

- Positive (n=1)
  - *B. pseudomallei*
    - Sputum culture positive (n=1)

- Negative (n=22)
  - *B. pseudomallei*
    - Sputum culture positive (n=2)

IFA performed (n=23)

- Positive (n=6)
  - *B. pseudomallei*
    - Sputum culture positive (n=3)

- Negative (n=17)
  - *B. pseudomallei*
    - Sputum culture positive (n=0)
Melioidosis cases n=89

- No stored serum sample n = 3
- Stored serum insufficient for AMD n = 15

Serum samples tested by AMD (n=71)

- Positive (n=5)
  - B. pseudomallei blood culture positive (n=5)
- Negative (n=66)
  - B. pseudomallei blood culture positive (n=31)