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MAJOR ARTICLE

What is the Role of Lateral Flow Immunoassay for the Diagnosis of Melioidosis?

Bart J. Currie,[1,2] Celeste Woerle,[1] Mark Mayo,[1] Ella M. Meumann,[1,2] and Robert W. Baird[2]

[1]Global and Tropical Health Division, Menzies School of Health Research, Charles Darwin University, Darwin, Northern Territory, Australia, and [2]Departments of Infectious Diseases and Pathology and Northern Territory Medical Program, Royal Darwin Hospital, Darwin, Northern Territory, Australia

Background. Culture of *Burkholderia pseudomallei* remains the gold standard for diagnosis of melioidosis but is not possible in many resource-limited settings where melioidosis is endemic. Direct identification of *B. pseudomallei* antigen in clinical samples has been developed using a lateral flow immunoassay (LFA) targeting *B. pseudomallei* capsular polysaccharide.

Methods. We summarized the findings from the 8 studies to date of the Active Melioidosis Detect (AMD) LFA and compared these with our results from 232 patients with culture-confirmed melioidosis. We have also optimized the methodology for testing different clinical samples.

Results. Sensitivity and specificity for different samples were broadly similar in our study to those published from Thailand, India, Laos, and Malaysia. One hundred thirty of 232 (56%) of our melioidosis patients were positive on 1 or more AMD tests: 27% for serum (rising to 39% in those with bacteremic melioidosis and 68% in those with septic shock), 63% for urine (72% in bacteremic melioidosis and 90% in septic shock), 85% in sputum that was culture positive, and 83% in pus that was culture positive. Heating sputum and pus samples increased sensitivity. Faint false-positive urine bands seen on earlier AMD versions were not seen when retested using the most recent version, AMD-Plus.

Conclusions. While the sensitivity of melioidosis LFA is low overall for blood samples, there is potential for use as a rapid diagnostic: testing serum and urine from those with severe sepsis who may have melioidosis and testing sputum and pus samples from clinically relevant scenarios. Prospective studies of patients with sepsis and other clinical presentations resembling melioidosis are required to ascertain if the specificity of AMD-PLUS is adequate to enable diagnosis of melioidosis with a high positive predictive value.

Keywords. *Burkholderia pseudomallei*; diagnostics; melioidosis; lateral flow immunoassay; rapid antigen test.

Culture of *Burkholderia pseudomallei* from a clinical sample remains the gold standard for diagnosis of melioidosis [1]. Without a confirmed culture, suspected cases of melioidosis remain simply "possible melioidosis." The emphasis on requiring a culture for a confirmed diagnosis is important for 3 reasons. First, from a therapeutic perspective, a confirmed diagnosis of melioidosis mandates a prolonged course of therapy; initially at least 10 days of intravenous ceftazidime or meropenem, followed by at least 3 months of oral eradication therapy, usually with cotrimoxazole (trimethoprim/sulfamethoxazole) [2]. Second, from an epidemiological and public health perspective, a confirmed diagnosis means that *B. pseudomallei* is present in the environment from which the patient was infected. This may expand knowledge of the global footprint of *B. pseudomallei,* with implications for empirical antibiotic guidelines for a newly recognized endemic focus of melioidosis, or it may alert public health authorities to potential imported melioidosis or even a biothreat scenario. Third, there remains no validated alternative means of making an accurate diagnosis of melioidosis, with positive serology potentially reflecting past rather than current infection [3], and nucleic acid amplification assays such as polymerase chain reaction (PCR) of clinical samples proving disappointingly inferior to culture [4].

The development of rapid antigen detection through lateral flow immunoassays (LFAs) and other antigen capture technologies has evolved rapidly over the last decade, with malaria and dengue being notably successful examples [5] and rapid antigen tests having an increasingly central role in the evolving global coronavirus disease 2019 pandemic [6]. Because the pathogen itself is being targeted in the tested clinical sample, a positive result should reflect active infection, which is a distinct advantage over serology. Nevertheless, specificity requires an antigen target that is unique to the pathogen being tested for and antigen capture methodology that will not capture/bind nonspecific targets.

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Correspondence: Bart Currie, FRACP, Menzies School of Health Research, PO Box 41096, Casuarina, NT 0811, Australia (bart.currie@menzies.edu.au).

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A diagnostic LFA for melioidosis has been developed [7] and deployed in several melioidosis-endemic regions for use in preliminary studies testing a variety of clinical samples [8–15]. The assay targets the *B. pseudomallei* capsular polysaccharide (CPS) [16] using a CPS-specific monoclonal antibody. Several modifications have been made to the assay since the prototype was evaluated. We have been optimizing the use of the LFA on a range of clinical samples and summarize the global experience together with our own findings and current standard operating procedures.

METHODS

The development of the prototype Active Melioidosis Detect (AMD) LFA has been described previously [7]. The assay involves the prepared clinical sample and a running buffer being applied to a test strip loaded with gold particles conjugated to *B. pseudomallei* CPS-specific monoclonal antibody. The complexes of antibody and bound *B. pseudomallei* CPS antigen from the clinical sample migrate up the strip by capillary action to be captured on a line of fixed *B. pseudomallei* CPS-specific monoclonal antibodies, giving a visible result. A positive control line is also included, using a nonspecific antibody. Since the development of the prototype AMD LFA, the manufacturers have made several alterations, including changing to a different *B. pseudomallei* CPS-specific monoclonal antibody. The most recent version, AMD-Plus, includes the addition of a proprietary inhibitor of nonspecific binding to address an issue of false-positive faint bands reported on some urine samples [11, 12].

We searched PubMed with the search terms "melioidosis" AND "lateral flow immunoassay" to identify articles describing the use of the LFA. We then summarized the findings from these studies, including test sensitivity and specificity where provided.

To these international data we added our own findings on the use of AMD on clinical samples from 232 patients with culture-confirmed melioidosis. We first summarized the overall sensitivity of AMD in patients with culture-confirmed melioidosis for each of the following sites: serum, urine, sputum, skin swabs, joint fluid, other pus/tissue, and cerebrospinal fluid. This analysis was on all clinical samples collected from the patients, whether or not the sample was culture positive for *B. pseudomallei.* We then from these data compared AMD results directly with culture results from the same patient for each of serum, urine, sputum, skin swabs, joint fluid, and other pus/tissue. Epidemiological, clinical, and laboratory data for these patients were collected as part of the Darwin Prospective Melioidosis Study, based at Royal Darwin Hospital, a 350-bed tertiary referral center in the tropical north of the Northern Territory of Australia [17]. In addition, we prospectively tested serial samples on 3 selected patients who had prolonged culture positivity for *B. pseudomallei* using AMD. AMD test kits were provided by the manufacturer (InBios International, Inc., Seattle, WA, USA).

Our final optimum methods for sample preparation and LFA testing with AMD-Plus are described in Supplementary Table 1 for each of serum, plasma, urine, sputum, pus, other tissue, swabs, culture bottles, and bacterial colonies from culture plates.

Patient Consent

Written informed consent was obtained for those having serial clinical samples collected beyond standard clinical management. This study was approved by the Human Research Ethics Committee of the Northern Territory Department of Health and the Menzies School of Health Research (HREC 02/38 and 04/09). InBios provided partial funding support for laboratory staff but were not involved in data analysis or manuscript preparation, and InBios had no editorial oversight.

RESULTS

International Studies

PubMed revealed 16 publications as of December 31, 2021, including the original description of AMD LFA from 2014 [7]. Of these, 8 assessed patients with possible melioidosis and calculated sensitivity and specificity of AMD using culture of *B. pseudomallei* as gold standard [8–15]. Table 1 summarizes these studies. The variety of clinical samples tested and analyses performed make direct comparisons between the study results problematic, but consistent themes emerged. First, the sensitivity on whole blood, serum, and plasma was very low overall and <50% even in bacteremic melioidosis but did increase in severe disease with presumed higher bacterial burden [9, 15]. Second, sensitivity was higher in sputum from those with melioidosis pneumonia and was mostly excellent in pus and fluids aspirated from abscesses in patients with melioidosis. Third, sensitivity in urine was high only when *B. pseudomallei* was cultured from urine (such as with genitourinary melioidosis), but urine AMD was sometimes also positive in those with more severe melioidosis but urine culture–negative for *B. pseudomallei*, presumably reflecting CPS urinary excretion. Fourth, while specificity overall was high, an issue of false-positive urine AMD with "faint +ve bands" was evident from 2 of the studies [11, 12] and resulted in the noted modification of the assay to the current AMD-Plus LFA. Finally, it was noted that collection and testing of serum, urine, and clinically relevant samples such as sputum and pus provided an overall sensitivity in melioidosis cases from "any LFA being positive" of 91% (21/23) in 1 study from India [14] and 65% (17/26) in 1 study from Laos [12]. These were small numbers from selected patients but supported further studies on the utility of AMD LFA.

The literature review also documented that AMD LFA is being increasingly used to diagnose melioidosis by directly

Table 1. Published Studies of Active Melioidosis Detect LFA

Author/Year (Reference)	Location Patient Cohort	Clinical Samples	Culture-Confirmed Melioidosis Patients, No.	Not Melioidosis, No.	AMD Sensitivity (Positive/Culture Positive for *B. pseudomallei*), %	AMD Specificity (Negative/Culture Negative for *B. pseudomallei*), %	Comment
Radhakrishnan 2021 [14]	Odisha, India Sepsis or community-acquired pneumonia	Serum, urine, sputum/throat swab, pus/body fluids	23	163	Serum 50% (10/20) Urine 52% (11/21) Sputum/throat swab 57% (4/7) Pus/body fluid 100% (9/9) Any AMD sample +ve from melioidosis patients 91% (21/23)	100% (15/15) 100% (30/30) Not available 100% (5/5) All AMD samples –ve from nonmelioidosis patients 100% (22/22)	Sensitivity calculated for all patients with melioidosis irrespective of which sample type was culture +ve, not by paired analysis of individual sample type culture results.
Amornchai 2021 [15]	Ubon Ratchathani, Thailand Community-acquired infections and sepsis	Serum	192	502	For all melioidosis patients Serum 31% (60/192) For bacteremic melioidosis 38% (57/150) For nonbacteremic melioidosis 7% (3/42)	99% (495/502)	Overlapping cohort with that reported in Wongsuvan 2018. Also showed AMD sensitivity higher with shorter duration of symptoms before diagnosis of melioidosis and with higher modified Sequential (sepsis-related) Organ Failure Assessment (SOFA) score.
Choi 2020 [13]	Sarawak, Malaysia Clinically possible melioidosis	Serum, urine, sputum, pus, body fluids	22 (blood and bodily fluid)	76	44%	98%	Sensitivity calculated by paired analysis of individual sample type culture results, but only collated results presented.
Rizzi 2019 [12]	Vientiane, Laos Clinically possible melioidosis	Whole blood, plasma, buffy coat, urine, sputum, pus	26	86	Whole blood 17% (2/12) Plasma 25% (3/12) Buffy coat 0% (0/12) Urine 67% (2/3) Sputum 80% (4/5) Pus 86% (6/7) Any AMD sample +ve from melioidosis patients 65% (17/26)	100% (94/94) 99% (93/94) 100% (94/94) 80% (74/93) 100% (23/23) 100% (13/13) All AMD samples –ve from nonmelioidosis patients 87% (75/86)	Sensitivity calculated by paired analysis of individual sample type culture results. "False +ve" AMD considered likely for 10 urine samples from patients without culture-confirmed melioidosis. All 10 showed only faint +ve bands.
Wongsuvan 2018 [9]	Ubon Ratchathani, Thailand Community-acquired infections and sepsis	Serum	192	566	For all melioidosis patients Serum 31% (60/192) For bacteremic melioidosis 37% (57/153) For nonbacteremic melioidosis 8% (3/40)	99% (559/566)	Overlapping cohort with that reported in Amornchai 2021.
Shaw 2018 [11]	Manipal, India Clinically possible melioidosis	Whole blood, urine, respiratory secretions, pus, body fluids	53	122	Whole blood 25% (2/8) Urine 100% (15/15) Respiratory secretions 100% (5/5) Pus 93% (27/29)	100% (1/1) 74% (26/35) 100% (40/40) 100% (43/43)	Sensitivity calculated by paired analysis of individual sample type culture results. "False +ve" AMD considered likely for 9 urine samples from patients without culture-confirmed melioidosis. All 9 showed only faint +ve bands.
Woods 2018 [10]	Vientiane, Laos Suspected melioidosis; diabetes and fever or sepsis; prostatitis; abscess in lung, liver, or spleen	Serum, urine, sputum, pus, body fluids	Not available	Not available	Serum 14% (5/36) Urine 67% (2/3) Selected urine 87% (13/15) Sputum 33% (1/3) Pus 47% (8/17) Serum +ve AMD in 7% (5/71) of melioidosis patients Urine +ve AMD in 18% (21/114) of melioidosis patients	Not available 100% (202/202) 91% (205/226) 100% (20/20) 100% (122/122)	Sensitivity calculated by paired analysis of individual sample type culture results.

Table 1. Continued

Author/Year (Reference)	Location Patient Cohort	Clinical Samples	Culture-Confirmed Melioidosis Patients, No.	Not Melioidosis, No.	AMD Sensitivity (Positive/Culture Positive for *B. pseudomallei*), %	AMD Specificity (Negative/Culture Negative for *B. pseudomallei*), %	Comment
Robertson 2015 [8]	Townsville, Australia Bacteremic melioidosis and other bacteremia controls	Whole blood (EDTA)	30	4	EDTA whole blood 40% (16/40)	100% (4/4)	40 samples from 30 bacteremic melioidosis patients; 4 samples from patients with bacteremia from another pathogen.

Abbreviations: AMD, Active Melioidosis Detect; LFA, lateral flow immunoassay.

testing turbid blood culture bottles or bacterial colonies growing on an agar plate [10, 11, 18]. AMD testing of bacterial colonies was used to help resolve an erroneous identification of *B. pseudomallei* as *B. thailandensis* by MALDI-TOF mass spectrometry [19]. One report documented preliminary work on the use of AMD for detection of *B. pseudomallei* from soil in environmental studies [20].

In addition, there was an informative report from India of 2 cases of melioidosis diagnosed by AMD on samples from endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) of necrotic mediastinal lymph nodes seen on chest computed tomography, where tuberculosis and lymphoma were possible diagnoses [21]. Therapy for melioidosis with meropenem was commenced in both cases on the day of the EBUS-TBNA after the AMD-positive result. In both cases, culture of *B. pseudomallei* subsequently confirmed the diagnosis, but in 1 the culture was only positive after 5 days.

Results From the Darwin Prospective Melioidosis Study

Of the 232 patients with culture-confirmed melioidosis who had clinical samples tested by LFA, 130 (56%) were positive by AMD from 1 or more clinical samples. Table 2 shows the AMD results for all clinical samples tested on all the confirmed melioidosis cases, irrespective of which clinical samples had been culture +ve and irrespective of timing of AMD samples in relation to culture samples from the patient, but removing any duplicate samples for an individual, such as where serial serum or sputum samples were collected. Table 3 compares AMD results with *B. pseudomallei* culture results for AMD tests done within 72 hours of the designated matched clinical sample culture. AMD testing was least sensitive on serum, with only 27% of all samples being positive by this method (Table 2), falling to 13% in patients who were blood culture negative for *B. pseudomallei* and rising to 39% in bacteremic patients and 68% in those with melioidosis septic shock (Table 3). Three of 4 fatal cases (75%) were serum AMD positive. Sputum was AMD positive in 48% of all samples and 85% of sputum culture–positive samples.

Table 2. AMD Results for Clinical Samples From 232 Patients With Culture-Confirmed Melioidosis

Clinical Sample[a]	No. Tested	No. AMD +ve (%)
Serum	172	47 (27)
Urine	129	81 (63)
Sputum	64	31 (48)
Skin swabs	38	17 (45)
Joint fluid	13	6 (46)
Other pus/tissue[b]	30	23 (77)
Cerebrospinal fluid[c]	9	3 (33)

Abbreviation: AMD, Active Melioidosis Detect.

[a]Only 1 sample per individual for each clinical specimen (where done), so duplicate samples such as serial serum, urine, or sputum were removed.

[b]Tissue samples included prostate, spleen, lung, brain, colon, bone, skin, lymph node.

[c]Only 1 cerebrospinal fluid sample was *B. pseudomallei* culture +ve.

Table 3. AMD Results for Matched Clinical Samples by Culture Results

Clinical Sample Tested[a] (Matched Patient and Culture Status)	No. Tested	No. AMD +ve (%)
Serum (blood culture –ve)	52	7 (13)
Serum (blood culture +ve)	62	24 (39)
Serum (septic shock[b] +ve)	19	13 (68)
Serum (fatal melioidosis)	4	3 (75)
Urine (blood culture –ve)	31	16 (52)
Urine (blood culture +ve)	25	18 (72)
Urine (septic shock[b] +ve)	10	9 (90)
Urine (urine culture –ve)	74	44 (59)
Urine (urine culture +ve)	19	15 (79)
Sputum (sputum culture –ve)	5	2 (40)
Sputum (sputum culture +ve)	27	23 (85)
Skin swabs (swabs culture +ve)	24	12 (50)
Joint fluid (fluid culture +ve)	8	5 (63)
Other pus/tissue (culture +ve)	24	20 (83)

Abbreviation: AMD, Active Melioidosis Detect.

[a]Only including those where AMD sample was taken within 72 hours of the matched culture sample. Only 1 sample per individual for each clinical specimen (where done), so duplicate samples such as serial serum, urine, or sputum were removed.

[b]Melioidosis septic shock, as defined in Currie et al. [17]. Serum and urine samples were taken within 72 hours of admission with septic shock.

Pus/tissue samples, joint fluid, and skin swabs were AMD positive in half or more samples where *B. pseudomallei* culture was positive. Cerebrospinal fluid was positive in 3 cases, only 1 of which was culture positive for *B. pseudomallei*, the only circumstance where AMD appeared more sensitive than culture.

Urine was AMD positive in 81/129 (63%) patients where a sample was collected. Urine AMD was positive in 79% of those who were urine culture positive for *B. pseudomallei*. Urine AMD was positive in 72% of those who had bacteremic melioidosis and 90% of those with melioidosis septic shock (Table 3).

False-Positive Urine AMDs

We compared AMD-Plus with earlier AMD LFAs on 9 urine samples from patients without melioidosis where a faint band was seen on the earlier LFA. The false-positive band was not seen on any of the 9 urine samples when retested using AMD-Plus on this limited number of samples (Figure 1).

Optimizing Sample Preparation and Use of Heat

As with previous studies [12], we found testing of serum and plasma to be more sensitive than buffy coat and whole EDTA blood (which can give indeterminate results including problematic color issues with the strip). We also found variable and inconsistent results on urine AMD when comparing whole urine with either sediment or supernatant from spun urine. Therefore, serum and whole urine are now our preferred samples for applying directly to the sample port of the test strip.

Various methods were trialed to address issues of viscous ("chunky") sputum, pus, and tissue samples resulting in failure of sample migration along the LFA test strip. For example, a higher concentration of lysis buffer showed improved results. Heating has been used to remove contaminants in the purification of CPS [22]. We found that heating sputum and pus samples increased the visual intensity of the AMD result, with some samples negative without heating being positive with the heating protocol (Figure 2; see Supplementary Table 1 for methods). Testing of control sputum and pus showed no false-positive results with the heating protocol (data not shown).

Serial AMD Sampling

Several patients with more severe disease and protracted positive *B. pseudomallei* cultures had persistently positive AMD serum, sputum, and/or urine samples. The longest durations were serum being positive for 109 days, sputum being positive for 163 days, and urine being positive for 179 days (Figure 3A–C).

DISCUSSION

LFA is a technology that is rapidly being adopted to fill a variety of diagnostic needs. LFA is an attractive point-of-care option because it is rapid and simple to use, providing a visual positive or negative result within 15 minutes. The nitrocellulose membrane on which antibodies and reagents are stored is robust in a range of climates and does not require refrigeration. As with culture of *B. pseudomallei,* LFA detects presence of the pathogen, but as with nucleic acid detection, it does not attest to organism viability. The major advantages of antigen detection over serology are that serology is often initially negative on presentation with melioidosis and that a positive serology result does not distinguish between melioidosis and past exposure to *B. pseudomallei,* as is commonly seen in melioidosis-endemic regions [3].

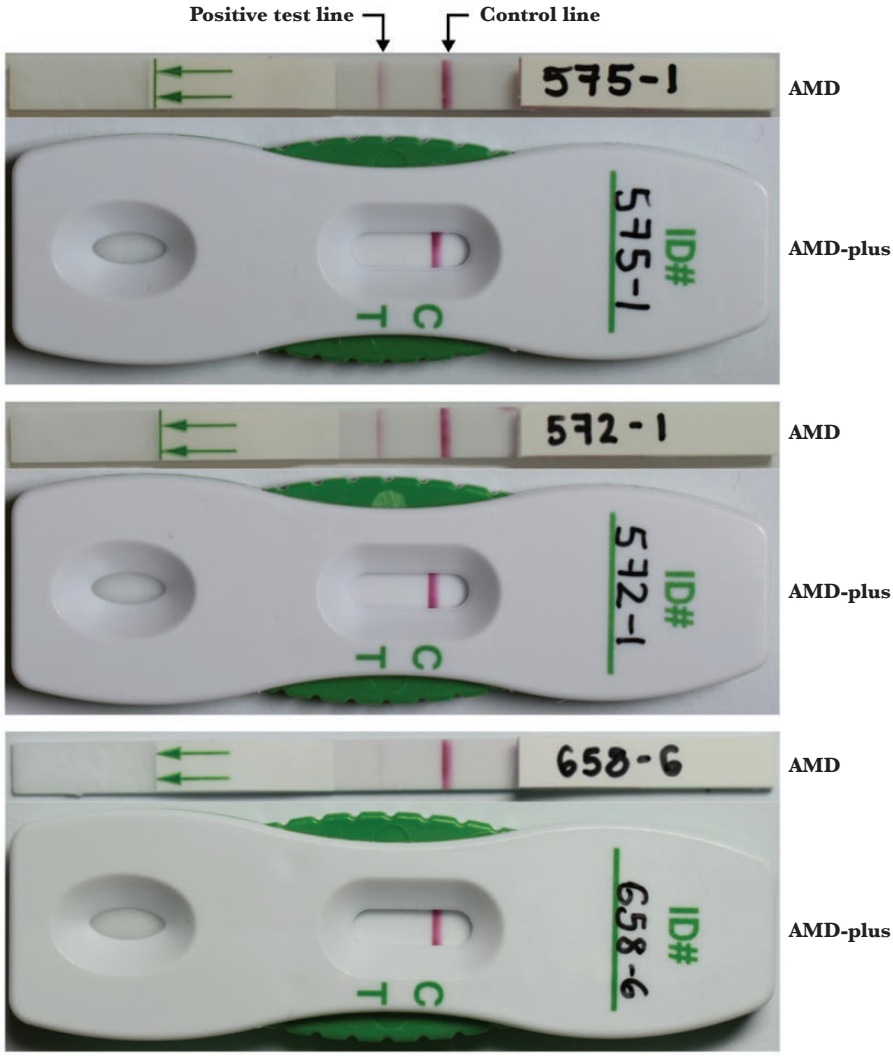

Figure 1. Three non-melioidosis patients with false-positive urine LFA AMD reversed when tested with AMD-Plus. Abbreviations: AMD, Active Melioidosis Detect; LFA, lateral flow immunoassay.

The results of our analysis of use of AMD LFA in 232 culture-confirmed melioidosis patients are broadly consistent with the published results from studies from other melioidosis-endemic regions. Across all our patients, representing the diversity of melioidosis presentations, AMD from at least 1 clinical sample was positive in 130/232, giving a sensitivity for diagnosis of melioidosis of 56%. This compares with AMD sensitivity of 65% in 26 patients from Laos with a diverse spectrum of culture-confirmed melioidosis who were similarly studied with AMD on a range of clinical samples including plasma, urine, sputum, and pus [12].

Our results confirm previous findings from Thailand that the sensitivity of serum/plasma using AMD increases substantially with severity of illness [9, 15]. This predominantly reflects the *B. pseudomallei* bacterial load in the bloodstream, but conceivably also higher tissue burdens of infection such as multiple lung and organ abscesses, with CPS likely to be shed into the bloodstream. Even in severe melioidosis, the bacterial load in blood is usually low, with quantitative bacterial counts showing 203 bacteremic patients in Thailand having a median *B. pseudomallei* count in blood (interquartile range) of 1.1 (0.2–7.7) CFU/mL [23]. This is a substantially lower count than patients with equivalent severity of sepsis from common pathogens such as *Staphylococcus aureus* and is reflected in blood cultures in patients with melioidosis often taking several days to become positive. In our study, AMD on serum was positive in only 13% of those who were blood culture negative, 39% of those who were blood culture positive, and 68% of those with melioidosis septic shock. Similar results from serum have been found in Thailand, with 7% and 38% AMD positive in those who were blood culture negative and positive, respectively [15]. In another study from Australia, AMD sensitivity on EDTA whole blood from 45 bacteremic melioidosis samples was 40% [8], but in a study from Laos only

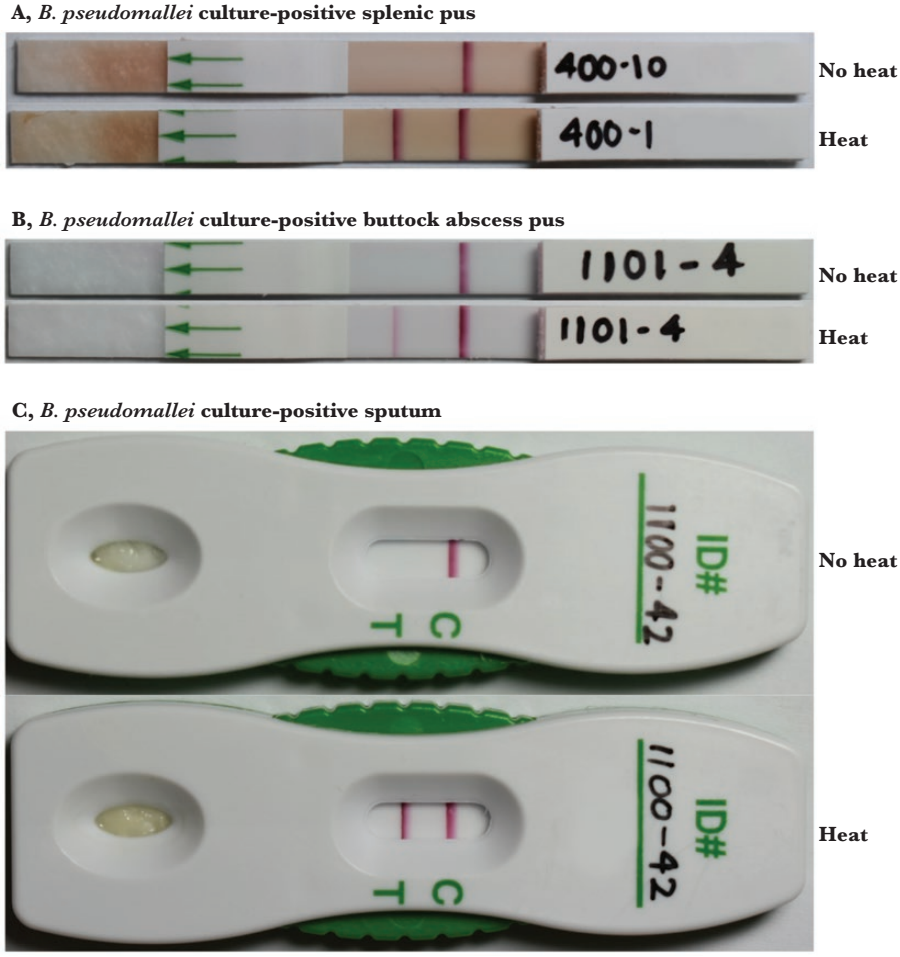

Figure 2. Heating improves LFA AMD sensitivity for pus and sputum. Abbreviations: AMD, Active Melioidosis Detect; LFA, lateral flow immunoassay.

14% of sera from bacteremic melioidosis patients were AMD positive [10].

A positive urine AMD can reflect either direct infection involving the genitourinary tract or antigenuria resulting from *B. pseudomallei* CPS shed into urine from blood. A mouse model showed *B. pseudomallei* CPS has a serum half-life of 2.9 to 4.4 hours, with CPS rapidly eliminated by renal filtration without degradation and despite its high molecular weight of 300 kDa [16]. In addition, there was no CPS accumulation in internal organs. Our finding of 79% of those who were urine *B. pseudomallei* culture positive being AMD positive compares with 80% in Laos melioidosis patients who were urine *B. pseudomallei* culture positive [10]. We also found 59% of melioidosis patients whose urine did not culture *B. pseudomallei* to be AMD positive, compared with 22% in Laos melioidosis patients whose urine did not culture *B. pseudomallei* [10]. That a positive urine AMD can reflect CPS antigenuria in addition to bacteriuria is supported by 72% of our patients with bacteremic melioidosis being urine AMD positive and the Laos study also finding higher positive urine AMD rates in those with more severe melioidosis [10]. In the Laos study, preliminary analysis of use of a table-top urinary concentrator before applying urine to the AMD strip showed potential for a moderate increase in sensitivity [10].

Previous studies have shown both sputum and pus to have higher AMD sensitivities than serum and urine. Our finding of 85% and 83% AMD positive for *B. pseudomallei* culture-positive sputum and pus samples, respectively, compares with 80% (sputum) and 86% (pus) from another Laos study [12] and 93% (pus) from India [11]. We have found AMD to be useful in many other tissue samples from patients with possible melioidosis: joint fluid, bone, spleen, lung, brain, cerebrospinal fluid, lymph node, skin (and skin swabs), and colon. Our finding that heating sputum and pus increases sensitivity is an observation that requires further validation on larger sample sets. *B. pseudomallei* CPS is stable at high temperatures [22], and heating potentially provides more access of the binding antibody to CPS through both CPS release from sputum and pus and CPS separation from other bacterial cell wall components.

Our data confirm all previous studies that, on a per-sample basis, culture of *B. pseudomallei* is more sensitive than AMD. However, AMD will detect CPS in some samples that are culture

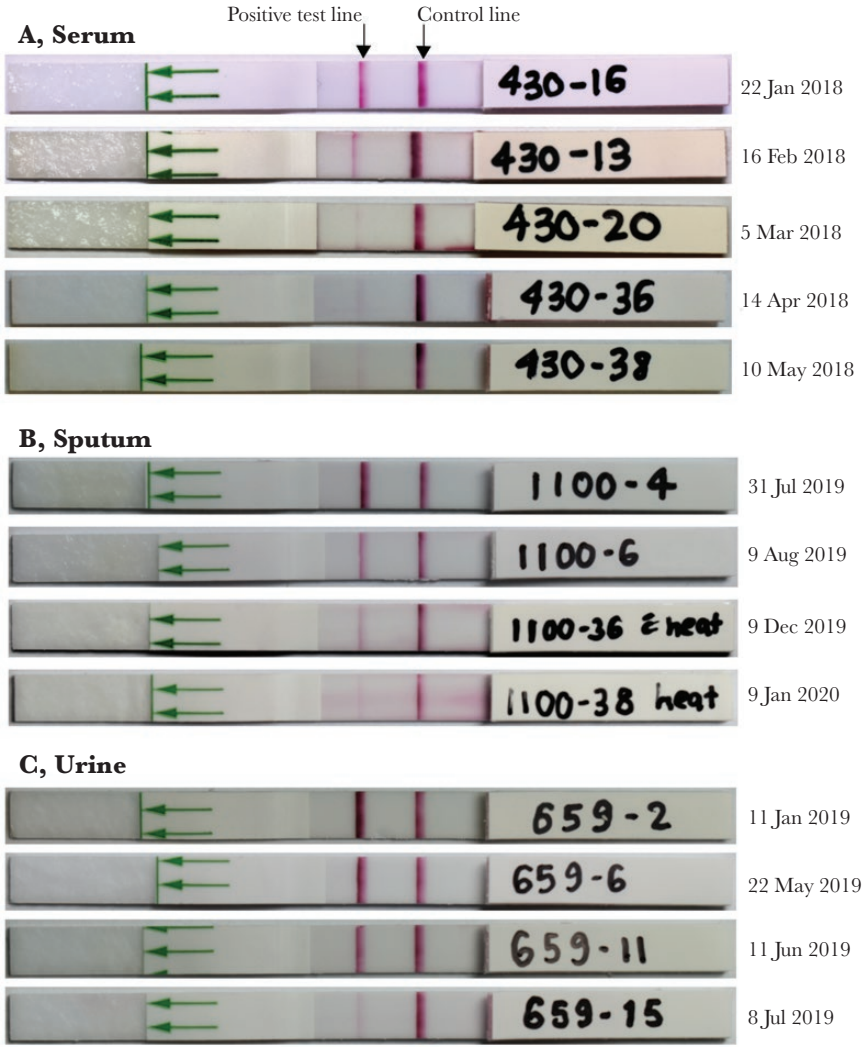

Figure 3. Prolonged serial positive LFA AMD results (A). Serum AMD positive for 109 days in a patient with melioidosis pneumonia and prolonged bacteremia (B). Sputum AMD positive for 163 days in a patient with cystic fibrosis and chronic pulmonary melioidosis (C). Urine positive for 179 days in a patient with severe bacteremic melioidosis pneumonia with mediastinal involvement. Abbreviations: AMD, Active Melioidosis Detect; LFA, lateral flow immunoassay.

negative. Samples that were culture negative and AMD positive tended to be from further into the admission and following the commencement of melioidosis-specific antibiotic treatment (ceftazidime or meropenem). This presumably reflects AMD detection of dead bacteria or CPS being shed into blood and urine during antibiotic treatment, which had substantially decreased or even completely cleared viable bacteria that were present before treatment.

The short half-life of *B. pseudomallei* CPS and its rapid excretion into urine suggest that a positive AMD from either serum or urine reflects active melioidosis infection and that serial AMD testing can potentially be used for monitoring clinical progress and response to therapy. This is supported by our serial sampling in a limited number of patients with more severe disease or who had protracted infection, such as chronic pulmonary melioidosis in cystic fibrosis. However, persistent AMD positivity may also reflect shedding of CPS from a large burden of already killed *B. pseudomallei,* and further studies are needed to correlate serial bacterial cultures with serial AMD testing.

A major benefit of AMD is that results are available within an hour or less of the clinical sample being collected. Even though AMD has only moderate sensitivity in comparison to cultures and culture does remain the gold standard for diagnosis of melioidosis, culture results in melioidosis are rarely available within 24 hours and can take several days or longer. Therefore, a positive AMD result for a patient with suspected melioidosis enables the clinician to both institute therapy targeting *B. pseudomallei* and undertake all the additional cultures and imaging appropriate for diagnosing melioidosis and the organs involved [21].

AMD is also being increasingly used to diagnose melioidosis by directly testing turbid blood culture bottles or bacterial colonies growing on an agar plate [10, 11, 18, 19]. AMD results have been generally similar to identification of *B. pseudomallei* using nucleic acid detection (PCR targeting the

TTSS1 region of *B. pseudomallei*) [24], latex agglutination [25], and direct immunofluorescence assay testing [26], all of which have both high sensitivity and specificity when used on blood cultures or colonies but which still require subsequent culture of *B. pseudomallei* for confirmation [1, 27]. Testing of many non-*pseudomallei Burkholderia* species and other near-neighbor species has shown the *B. pseudomallei* CPS antigen targeted by AMD to be highly specific to *B. pseudomallei* [7]. The only exception found to date is a small cluster of variant *B. thailandensis* isolates that exhibit isolated acquisition of the CPS biosynthetic operon, which results in a positive AMD test [28]. Of these, E555, which is an environmental *B. thailandensis* from Cambodia, has been shown to be avirulent in mice. Therefore, such strains are very unlikely to be encountered in the clinical setting of using AMD for testing patients with suspected melioidosis. However, the issue of false-positive AMD results from environmental non-*pseudomallei Burkholderia* species may be encountered when AMD is used to detect environmental *B. pseudomallei* in soil and water studies. It has been proposed that in resource-limited settings, soil samples positive with AMD could then be sent to reference laboratories for confirmation with culture, thus facilitating the expansion of the global risk map for melioidosis [20]. However, further validation of the use of AMD on environmental samples will be required before widespread deployment.

The major limitation of the results from the Darwin study is that we are only describing AMD results for culture-confirmed melioidosis patients, and we are therefore focusing on sensitivity of the assay and not specificity. Specificity of AMD has been described in previous studies [8–15], with the proviso that culture of *B. pseudomallei* as the gold standard reference is not perfect, with 1 Bayesian analysis estimating the true sensitivity of culture for melioidosis to be only 60.2% [29]. However, we suggest that this figure of 60.2% sensitivity of culture is likely to be a substantial underestimate of the true sensitivity of the use of bacterial culture for diagnosis of melioidosis in Darwin and in other circumstances where laboratory and clinical resources enable complex imaging and diagnostic procedures, with multiple cultures taken from multiple sites and repeated when diagnosis remains unconfirmed.

In general, specificity of all clinical samples with AMD has been excellent in prior studies, with the exception that faint positive bands seen on urine samples in published studies have been thought likely to reflect truly false-positive urine results [11, 12]. This was the rationale for the manufacturer adding a proprietary inhibitor of nonspecific binding to the latest generation of AMD, AMD-Plus. Our limited testing of this AMD-Plus version showed removal of the false-positive bands seen in some urine samples (Figure 1), but larger studies are required to confirm this specificity improvement.

The importance of specificity approaching 100% for any melioidosis diagnostic cannot be overemphasized. A diagnosis of melioidosis results in prolonged therapy for the individual and may also have epidemiological and public health implications if the case is from an unexpected geographical location not known to have endemic melioidosis [30]. For example, if prevalence of melioidosis in the patient cohort tested (eg, sepsis patients in the emergency department) is 5%, then an AMD specificity of 95% means that for every 1 true melioidosis case diagnosed you can expect 1 false-positive diagnosis.

Another limitation of comparing AMD results from all international studies and combining the Darwin AMD results over time is that these combined results are from all the generations of AMD, rather than comparing results across generations. Nevertheless, both the international studies and the Darwin study found sensitivity to be broadly conserved between the generations of the AMD assay, and combining results in the Darwin study was the only way to have adequate numbers to assess the sensitivity of AMD across a variety of clinical sample types.

In conclusion, LFA with AMD has several useful roles for presumptively diagnosing melioidosis from selected clinical samples. Sensitivity is higher for pus and sputum than for serum and urine, but we found that a presumptive diagnosis of melioidosis can be made in over half of all cases when AMD is used on multiple case-specific appropriate clinical samples. A positive urine AMD can also reflect antigenuria from CPS, which is rapidly filtered from the blood. Sensitivity rises with bacterial load, such that urine was AMD positive in 72% of those with bacteremic melioidosis and serum was AMD positive in 65% of those with melioidosis septic shock. Serial AMD results on sputum, serum, and urine provide insights into treatment progress. Specificity of AMD has been improved with modifications in the most recent test kit, AMD-Plus. Large prospective studies of patients presenting with possible melioidosis are now required across several melioidosis-endemic regions to accurately quantitate the specificity of AMD. This will establish the accuracy of a diagnosis of melioidosis using AMD and, thereby, if AMD can be a useful and safe test even in areas where incidence of melioidosis is low. Meanwhile, culture of *B. pseudomallei* remains the required standard for any confirmation of a diagnosis of melioidosis.

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