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1 **Evaluation of a rapid diagnostic test for the detection of *Burkholderia***

2 ***pseudomallei* in the Lao People's Democratic Republic**

3

4 Running title

5 Melioidosis diagnosis by rapid antigen detection Laos

6

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23 **Abstract**

24 *Burkholderia pseudomallei* causes significant global morbidity and mortality,
25 with the highest disease burden in parts of Asia where culture-based
26 diagnosis is often not available. We prospectively evaluated the Active
27 Melioidosis Detect (AMD, InBios International, USA) lateral flow immunoassay
28 (LFI) for rapid detection of *B. pseudomallei* in turbid blood cultures, pus,
29 sputum, sterile fluid, urine, and sera. Performance was compared to *B.*
30 *pseudomallei* detection using monoclonal antibody latex agglutination (LA)
31 and immunofluorescence assays (IFA), with culture as the gold standard.
32 AMD was 99% (99/100; 94.6 – 100%) sensitive and 100% (308/308; 98.8-
33 100%) specific on turbid blood culture bottles, with no difference to LA or IFA.
34 AMD specificity was 100% on pus (122/122; 97.0-100%), sputum (20/20;
35 83.2-100%), and sterile fluid (44/44; 92 – 100%). Sensitivity on these samples
36 was: pus 47.1% (8/17; 23.0 – 72.2%), sputum 33.3% (1/3; 0.84 – 90.6%), and
37 sterile fluid 0% (0/2; 0 – 84.2%). Urine AMD had a positive predictive value of
38 94% (32/34; 79.7 – 98.5%) for diagnosing melioidosis in our cohort. AMD
39 sensitivity on stored sera, collected prospectively from melioidosis cases
40 during this study, was 13.9% (5/36; 4.7% - 29.5%) when compared to blood
41 culture samples taken on the same day. In conclusion, the AMD is an
42 excellent tool for rapid diagnosis of melioidosis from turbid blood cultures, and
43 maintains specificity across all sample types. It is a promising tool for urinary
44 antigen detection, which could revolutionise diagnosis of melioidosis in
45 resource-limited settings. Further work is required to improve sensitivity on
46 non-blood culture samples.

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47 **Introduction**

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

61

62 Culture is currently the gold standard diagnostic test for melioidosis with 100%
63 specificity but an estimated sensitivity of only 60% (7). However, culture
64 confirmation takes a minimum of 48-72hrs (7), requires specific media for
65 optimal sensitivity in non-sterile samples (8), and laboratory containment and
66 expertise often not available in endemic areas. *B. pseudomallei* is intrinsically
67 resistant to many antibiotics and melioidosis therefore does not respond to
68 most agents used for empiric treatment of sepsis, pneumonia and abscesses
69 in developing countries (9). Therefore, life-saving treatment is often fatally
70 delayed if a specific diagnosis cannot be confirmed. Simple, rapid diagnostic
71 tests for melioidosis for use directly on clinical samples are needed, not only

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72 for improving patient outcomes but also to improve epidemiological
73 surveillance of melioidosis and thereby strengthen public health interventions.
74 Immunofluorescence to detect *B. pseudomallei* directly in clinical samples
75 (10) and latex agglutination for rapid identification of positive cultures (11, 12)
76 are used in some melioidosis-endemic areas. However, these require
77 equipment, expertise and reagents that are not widely available. More
78 recently, an immunochromatographic lateral flow rapid diagnostic test (RDT)
79 to detect *B. pseudomallei* extracellular polysaccharide antigen directly in
80 clinical samples has been developed (Active Melioidosis Detect (AMD),
81 InBios, USA) (13). The method is simple, rapid (result within 15 minutes),
82 relatively cheap (estimated $2/test), does not require additional equipment,
83 and the kit may be stored at room temperature, making it ideal for use in
84 resource-limited settings. To date this kit has undergone only limited clinical
85 evaluations (13, 14). We evaluated the diagnostic performance of AMD on a
86 variety of clinical samples (including blood culture broths, pus, sterile fluid,
87 sputum, and urine) over two rainy seasons from patients with suspected and
88 culture-confirmed melioidosis presenting to Mahosot Hospital, Vientiane,
89 Laos.
90

91 **Results**

92 <u>Analytical sensitivity and specificity</u>

93 All *B. pseudomallei* seeded blood cultures were AMD-positive by 12hrs
94 incubation (Table S1). The lower limit of detection of the AMD was found to be
95 1.4 x 10^5 CFU/ml, meaning that it was positive in 3 of 4 seeded blood cultures
96 before they became visibly turbid and in 4/4 before organisms were

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97 detectable by Gram staining of the broth. Centrifugation of blood culture broth
98 prior to performing AMD did not reduce time to positivity.
99
100 All non-*B. pseudomallei* reference strains were negative by AMD (Table S2).
101 Two soil isolates of *B. thailandensis* and one sputum isolate of *B. cepacia*
102 were AMD-positive. All 3 of these isolates were known to give a positive *B.*
103 *pseudomallei* latex agglutination test, probably due to production of a cross-
104 reacting extracellular polysaccharide (15). All four positive control *B.*
105 *pseudomallei* clinical isolates were positive by AMD.
106
107 <u>Prospective evaluation</u>
108 Between 26th June and 18th December 2014, 89 patients were diagnosed with
109 melioidosis by culture of *B. pseudomallei* from at least one sample type
110 (blood, pus, sputum, sterile fluid, urine, throat swab, wound swab (Figure
111 S1)). Median age was 45 years (IQR 27-54 years), 55% were male and 20/89
112 (22.5%) patients died during admission or were discharged moribund. Blood
113 cultures were received from 85 of these patients (85/89, 96%) of whom 54%
114 (46/85) were bacteraemic with *B. pseudomallei.* Of the 43 patients not found
115 to be bacteraemic, 34 had localized disease whilst 9 had evidence of
116 multifocal disease.
117
118 *Turbid blood cultures*
119 There were 412 turbid blood culture bottles during the study period, of which 4
120 did not have AMD performed and were therefore excluded. 408 turbid blood
121 culture bottles from 247 patients were thus included in the analysis.

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122 Organisms isolated from the 408 blood cultures are shown in Figure S2. *B.*
123 *pseudomallei* was isolated from 100 bottles (Figure 1a). Overall AMD
124 sensitivity was 99% (99/100; 94.6 – 100%) and specificity 100% (308/308;
125 98.8-100%). 252 of these 408 turbid blood culture bottles had Gram-negative
126 bacilli (GNB) seen on Gram stain and therefore had additional rapid tests
127 performed: latex agglutination (n=237), immunofluorescence assay (IFA;
128 n=176) (Figure 1a). Sensitivities and specificities for these tests are given in
129 Table 1. 166 turbid blood culture samples with GNB seen on microscopy had
130 all 3 rapid tests performed, with agreement between all 3 rapid tests and
131 culture in 98.2% (163/166). 1 sample was AMD and latex agglutination
132 negative but IFA and *B. pseudomallei* culture positive. 2 samples were scanty
133 positive by IFA (1-3 bacilli/100 fields) but negative by all other tests.
134
135 *Pus*
136 139/150 pus samples received during the study period had AMD performed
137 and were included in the analysis. *B. pseudomallei* was cultured from 17/139
138 samples. 95/139 pus samples also underwent IFA (Figure 1b). AMD was
139 significantly more specific than IFA (p=0.0006; Table 1). However, all 8 false
140 positive IFA results were reported as scanty (1-5 bacilli/100 fields).
141
142 *Sputum*
143 26 sputa were received during the study period, 3 of which did not have AMD
144 performed and were excluded. The remaining 23 sputa underwent both AMD
145 and IFA testing and no significant difference in sensitivity or specificity was
146 found (Table 1). 3/23 samples were *B. pseudomallei* culture positive, all 3 of

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147 which were IFA positive but only 1 of which was AMD positive (Figure 1c).
148 There were 3 positive IFA tests from culture negative samples, but all were
149 reported as scanty (1-7 bacilli/100 fields), and 2 of these samples were from
150 the same patient who, although culture negative for *B. pseudomallei*, had a
151 clinical picture compatible with melioidosis and died shortly after transfer to
152 Thailand for further healthcare.
153
154 *Sterile Fluid*
155 Between 10th October and 18th December 2014 50 sterile fluid samples were
156 received, 46 underwent AMD and were included in the analysis (29 pleural
157 fluid; 6 joint fluid; 2 pericardial fluid; 9 ascitic fluid). 42/46 also underwent IFA.
158 *B. pseudomallei* was isolated from 2 samples (both joint fluid from the same
159 patient, samples taken 11 days apart); IFA was positive on both these
160 samples whilst AMD was negative on both (Figure 1d). The sensitivity of IFA
161 was therefore significantly better than AMD (Table 1) although numbers are
162 small, and confidence intervals wide.
163
164 *Urine*
165 Between 2nd July and 2nd Sept. 2014 249 urine samples were received, AMD
166 was not performed on 28 and 16 were duplicate specimens, thus 205 were
167 included in the analysis (Figure 1e.i). 3/205 urine samples were *B.*
168 *pseudomallei* culture positive, 2 of which were AMD positive (sensitivity 66.7%
169 (9.4 - 99.2%)). The *B. pseudomallei* culture positive urine sample which was
170 AMD negative had a low bacterial load with only 1 colony forming unit isolated
171 from a centrifuged deposit. AMD specificity was 100% (98.2 - 100%;

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172 202/202). Organisms isolated in culture from all 205 urines are shown in
173 Figure S3.
174 From 3rd September onwards only selected urines were included in the study
175 (3rd Sept. - 18th December 2014 n=102; 23rd June – 12th Nov. 2015 n=189).
176 AMD was not performed on 27 samples (2014: 5; 2015: 22 samples), and 23
177 samples were duplicates and therefore excluded. Thus 241 samples were
178 included in the analysis. 15/241 urine samples were *B. pseudomallei* culture
179 positive, 13/15 of which were AMD positive (Table 1). 21/226 urine culture
180 negative samples were AMD positive (Figure 1e.ii). Interestingly 19/21 of
181 these urines came from patients who had melioidosis confirmed by culture
182 from another site suggesting that these were not "false positive" AMD results
183 but that the AMD was detecting true *B. pseudomallei* antigenuria. The positive
184 predictive value of AMD on urine for correctly diagnosing melioidosis in this
185 cohort was therefore 94.1% (32/34; 79.7 – 98.5%) with a disease prevalence
186 of 35.7% (86/241).
187 *Urine samples from melioidosis cases.* To further describe *B. pseudomallei*
188 antigenuria in our cohort results of urine samples received from the 182
189 culture-confirmed (from any site) melioidosis cases during the 2014 and 2015
190 study periods were analyzed further. A urine sample was received from 114
191 (2014: 57; 2015: 57) of these 182 patients, 20/114 urines were culture positive
192 for *B. pseudomallei* (16/20 were AMD positive). 21 of the 94 culture-negative
193 urines were AMD positive (Table 2). Patients who were urine culture negative
194 for *B. pseudomallei* but had disseminated melioidosis were significantly more
195 likely to be urine AMD positive than those with localized melioidosis (18/61 vs
196 3/32; p = 0.036). Presence of *B. pseudomallei* bacteraemia did not increase

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197 the likelihood of urine AMD positivity overall (15/49 vs 6/44, p = 0.08), or in
198 patients with disseminated melioidosis (15/49 vs 3/12, p = 1.0).
199
200 *Urine concentration.* Due to lack of availability of urine concentrators this was
201 performed retrospectively on 20 stored urine isolates collected prospectively
202 between 2nd July and 18th December 2014. All urine samples were from
203 confirmed melioidosis cases but were AMD negative on neat urine and *B.*
204 *pseudomallei* urine culture negative. 6 of 20 urine samples were AMD positive
205 after urine concentration.
206
207 *Sera*
208 71 stored serum samples from the 89 melioidosis cases diagnosed from 26th
209 June – 18th December 2014 were available for AMD testing (Figure 1f). 5/71
210 (7%) samples were AMD positive. Each of these five patients were culture-
211 positive for *B. pseudomallei* from blood taken on the same day as the serum
212 sample. Of the patients negative by AMD on sera, 31/66 had been
213 bacteraemic with *B. pseudomallei* on the same day as the serum sample was
214 taken, and a further 9 non-bacteraemic patients had evidence of disseminated
215 disease. Sera therefore had low sensitivity for diagnosis of melioidosis when
216 compared with blood culture as the gold standard, 13.9% (5/36; 4.7% -
217 29.5%).
218
219 **Discussion**
220 We evaluated accuracy of the AMD lateral flow immunoassay (LFI) for the
221 rapid diagnosis of melioidosis directly from clinical samples. The LFI detects

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222 extracellular polysaccharide of *B. pseudomallei* with a limit of detection (LOD)
223 of 0.2ng/mL (13, 16). On turbid blood cultures the AMD was found to have
224 excellent analytical and diagnostic sensitivity and specificity, comparable to
225 both IFA and latex agglutination which were found to have similar
226 performance characteristics to previous studies (11, 17-19). One turbid blood
227 culture bottle was AMD and latex agglutination negative but culture positive
228 for *B. pseudomallei*. After 24hours further incubation both tests were positive
229 on the same bottle suggesting that the initial false negative results may reflect
230 an initial bacterial load lower than the limit of detection for both tests. The high
231 specificity of the AMD on turbid blood cultures is extremely promising for
232 deployment to field settings in the tropics, where simple tests such as the
233 AMD may be performed after a broth incubation step in the absence of
234 laboratory facilities for Gram stain and culture. A positive AMD result,
235 potentially obtained as early as 12 hours post blood sampling, could prove
236 life-saving if antimicrobial treatment is adapted appropriately.
237 The AMD maintained excellent analytical and diagnostic specificity across all
238 sample types, even 'non-sterile' samples such as sputum and urine, which are
239 more likely to contain contaminating bacteria (including environmental
240 *Burkholderia* spp. in an endemic tropical setting). False positive AMD results
241 were only seen in our study with environmental *Burkholderia* strains known to
242 express a similar extracellular polysaccharide to that detected by the AMD.
243 Although this may pose a problem when the AMD is used for detection of *B.*
244 *pseudomallei* in environmental samples such as soil (20), in clinical samples it
245 is rarely likely to be relevant. The finding of AMD reactivity in a clinical strain
246 of *B. cepacia* in this study is novel, as previous testing of *B. cepacia* complex

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247 strains had not demonstrated this (unpublished observations, D. AuCoin).
248 Relatively few sputum samples were included in this study and further work is
249 needed to investigate how frequently respiratory samples containing non-
250 *pseudomallei Burkholderia* (for example from patients with cystic fibrosis) may
251 cause AMD reactivity. However, in our experience such strains are rare and
252 the majority of *B. cepacia* isolates do not cross react in this way (data not
253 shown).
254 AMD analytical sensitivity was extremely promising, with AMD LOD on broth
255 cultures better than previous estimates of LOD for latex agglutination (1 – 2
256 $x10^6$ CFU/ml (18)), and similar to estimated bacterial loads of *B.*
257 *pseudomallei* in urine (1.5 x 10^4 CFU/ml), sputum (1.1 x 10^5 CFU/ml), and pus
258 ($1.1x10^7$ CFU/ml) samples (21). However, diagnostic sensitivity was
259 disappointing in non-blood culture samples. We observed that viscous
260 samples were more challenging to process for AMD and this, coupled with low
261 initial bacterial loads, may explain the moderate sensitivity observed in these
262 samples. Seven pus samples in our study were received in broth from distant
263 study sites (and therefore excluded from the main analysis) and AMD was
264 positive in all 3 samples subsequently *B. pseudomallei* culture positive. An
265 initial enrichment culture step has been previously suggested to improve the
266 sensitivity of IFA for *B. pseudomallei* from non-blood clinical samples (10). It is
267 likely that broth incubation of pus, sputum and sterile fluid found to be AMD
268 negative on direct testing will increase AMD sensitivity on these samples,
269 whilst only delaying diagnosis by a few hours, although appropriate laboratory
270 bio-safety equipment and practices would be needed.

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271 IFA diagnostic sensitivity and specificity in this study were similar to previous
272 reports (10). However, despite the reportedly lower LOD ($2x10^3$ CFU/ml of
273 IFA (10), IFA was not found to be significantly more sensitive than AMD on
274 any sample type except sterile fluids, nor was IFA significantly more specific
275 than AMD except on pus samples. The inherent subjectivity of
276 immunofluorescent microscopy, the difficulty of misidentifying fluorescent
277 debris as bacteria, and the labour and resource-intensive methodology are
278 important disadvantages of IFA compared with AMD.
279 The number of *B. pseudomallei* culture-positive urine samples in this study
280 was limited, however the ability of the AMD to detect *B. pseudomallei*
281 antigenuria in melioidosis patients, particularly those with disseminated
282 melioidosis whose urine is culture-negative for *B. pseudomallei*, is
283 encouraging. Our findings replicate previous work in a non-human primate
284 model, in which antigen was detectable in urine by AMD as early as 2-3 days
285 after experimental infection with *B. pseudomallei* (D. AuCoin personal
286 communication). The extracellular polysaccharide detected by the AMD
287 maintains its molecular weight in urine without degradation over time which
288 might otherwise affect the sensitivity of the AMD on urine (22). Urine is an
289 easily available, non-invasive sample and a simple matrix for the AMD.
290 Reliable urine antigen detection for melioidosis by a rapid test such as the
291 AMD could revolutionize diagnostics in resource-limited parts of the world
292 where this disease is most prevalent. However, the overall sensitivity of urine
293 AMD for detection of melioidosis in our cohort was only 33% (37/114; 24 –
294 41.9%). Use of simple table-top urine concentrators increased AMD sensitivity
295 in this study, however numbers tested were limited and further work is needed

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296 to establish the extent to which urine concentration improves AMD sensitivity
297 in urine. At approximately $6/ sample, cost may prove a barrier to the use of
298 this concentration technique for urine samples in endemic settings.

299 The AMD is an extremely promising tool for diagnosis of melioidosis
300 worldwide and meets all ASSURED criteria for RDTs (23), although further
301 work is required to optimize sensitivity on non-blood samples. However, it is
302 not yet a true 'Point of Care' test, with AMD sensitivity on stored whole blood
303 samples from bacteraemic melioidosis patients having previously been shown
304 to be 40% (16/40; compared with 20% sensitivity for molecular detection on
305 the same samples) (14), and on stored serum samples in this study only 7%
306 (5/71). The low bacterial load in blood samples (1 CFU/ml prior to broth
307 incubation (13)) may limit the utility of the AMD on these sample types and
308 further large-scale prospective evaluations are required. However, the simple
309 nature of the AMD technology means that it is more likely than molecular
310 diagnostics to be widely applicable in a developing country context for the
311 foreseeable future.

312 The main limitation of this study was that not all samples underwent testing by
313 all the relevant rapid tests. This was partly related to the nature of the study
314 with the assays being performed during routine processing in a busy
315 diagnostic laboratory. However, this study design gave a realistic indication of
316 assay performance in a routine setting. In addition, the immunofluorescence
317 assay, which had not previously been routinely used in our laboratory, took
318 longer than expected to optimize and therefore turbid blood cultures and pus
319 received in the initial stages of the study did not undergo IFA. Sputum and
320 sterile fluid analyses were also limited due to low sample numbers.

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321 In some patients in our study a positive urine AMD result was the first
322 indication of melioidosis, preceding culture confirmation by at least 24hrs and
323 resulting in early switch to appropriate antibiotics in critically unwell patients.
324 This study was not designed to evaluate the clinical impact of obtaining a
325 rapid diagnosis of melioidosis by AMD, however now that we have shown
326 diagnostic specificity to be excellent, we are further investigating this. Throat
327 swabs are another easily obtained sample type that is routinely used for
328 diagnosis of melioidosis by culture in our setting. Incubation of a throat swab
329 in liquid selective enrichment broth such as SBCT (8) would enable throat
330 swabs to be used for rapid diagnosis of melioidosis by AMD, and this also
331 warrants further study.
332 In conclusion, the AMD has excellent sensitivity and specificity for early
333 detection of *B. pseudomallei* in blood culture broth. It also has the advantage
334 over the latex agglutination test that it can be used directly on other sample
335 types for the diagnosis of melioidosis. Specificity is retained when used
336 directly on these other samples, but sensitivity is only moderate and requires
337 optimization. Diagnosis of melioidosis from urine using AMD may significantly
338 enhance diagnosis of this neglected disease. Studies are needed in a variety
339 of different prevalence settings in order to truly understand the utility of this
340 assay, however deployment of the AMD globally could improve our
341 understanding of the epidemiology of melioidosis. Ultimately, we hope to see
342 LFI technology used to multiplex antigen detection for a number of important
343 causes of febrile illness in resource-limited settings.

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344 **Materials and Methods**

345 Analytical Sensitivity and Specificity

346 The limit of detection of the AMD was assessed using seeded blood cultures
347 and quantitative culture techniques. A single colony of *B. pseudomallei*
348 (clinical isolate) was taken from a 24-48 hour culture and a 0.5 McFarland
349 suspension made using 5ml phosphate buffered saline (PBS). This was
350 diluted to 1:1,000,000 and then 1 ml inoculated into each of 4 negative blood
351 culture bottles (Pharmaceutical factory No. 2, Vientiane, Laos; (24)), shaken
352 gently to mix and incubated aerobically at 35-37°C. From time of inoculation
353 (Time 0) and 12 hourly thereafter blood culture broths were observed for
354 turbidity, Gram stain was performed using standard methods, and AMD and
355 quantitative culture were performed. AMD was performed in duplicate on
356 paired uncentrifuged and centrifuged samples from each blood culture broth
357 as follows: 0.5ml of blood culture broth mixture was dispensed into a 1.5ml
358 Eppendorf tube, 1 drop of lysis buffer was added and the suspension gently
359 mixed using a micropipette. 20µl of this suspension was then applied to the
360 AMD test strip, followed by 3 drops of chase buffer. The result was read after
361 15 minutes according to manufacturers instructions (Appendix 1). For
362 centrifuged samples 1ml of blood culture broth was centrifuged at 845*g*
363 (3000rpm on Microcentrifuge 5424 Eppendorf) for 10 minutes, the pellet then
364 re-suspended in 1 drop of lysis buffer and 20µl of this suspension applied to
365 the AMD test strip and read as above. All centrifugation was carried out in
366 Biosafety Containment Level 3.

367 Quantitative culture was performed using the Miles and Misra method (25):
368 1ml was removed from each of the seeded blood culture broths after 0, 12

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369 and 24hrs of incubation. Each 1ml was diluted 1:10 in PBS 8 times to make
370 dilutions of 10^{-1} to 10^{-8} concentration. 20µl of each of these dilutions was
371 pipetted onto 1/6th of a blood agar plate, allowed to dry for 20-30 minutes,
372 and then incubated aerobically at 35-37°C for 24hours. Each dilution was
373 tested in triplicate. Colony-forming units/ml (CFU/ml) were calculated for each
374 seeded blood culture as: average number of colonies for the dilution where
375 the highest numbers of discrete colonies (between 10-100) were clearly seen
376 x 50 x dilution factor. The result of quantitative culture was recorded and
377 compared to AMD positivity across the panel of 4 seeded blood cultures to
378 estimate AMD limit of detection.
379
380 To investigate analytical specificity, negative blood culture broths were
381 seeded (as above) with a range of NCTC/ATCC reference organisms
382 (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Klebsiella*
383 *oxytoca* NCTC 8167, *Enterobacter aerogenes* NCTC 10006, *Enterobacter cloacae*
384 NCTC 11580, *Citrobacter freundii* NCTC 9750, *Edwardsiella tarda* NCTC 10396,
385 *Salmonella* Typhi NCTC 786, *Salmonella* Enteritidis ATCC 13076, *Pseudomonas*
386 *aeruginosa* ATCC 27853, *Acinetobacter baumannii* NCTC 12156, *Ochrobactrum*
387 *anthropic* NCTC 12168, *Aeromonas hydrophila* NCTC 8049, *Yersinia enterocolitica*
388 NCTC 11175, *Vibrio cholera* NCTC 8021, *Burkholderia thailandensis* NR-9908,
389 *Burkholderia cepacia* NCTC 10743, *Staphylococcus aureus* ATCC 29213,
390 *Staphylococcus epidermidis* NCTC 11047), soil isolates of *Burkholderia*
391 *thailandensis,* and Lao clinical isolates of *B. pseudomallei* and *B. cepacia*
392 (Table S2). Broths were incubated aerobically at 35-37°C and observed daily
393 for turbidity. AMD was performed on uncentrifuged samples from turbid blood
394 culture broths (as previously described).

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396 Prospective Evaluation

397 *Study population*

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

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

412

413 *Sample processing*

414 All samples were processed for microscopy and culture according to standard
415 laboratory procedures (CLSI). *B. pseudomallei* was isolated from clinical
416 samples using standard (goat blood, chocolate and MacConkey agars),
417 and specific selective media (Ashdown's agar and modified Ashdown's
418 selective broth (8)). Each set of blood cultures sent from a patient at any one
419 time contained two blood-broth culture bottles (Pharmaceutical factory No. 2,

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420 Vientiane, Laos; (24)), which were incubated aerobically at 35-37°C and
421 observed daily for turbidity for up to 7 days.
422
423 *Blood cultures:* AMD was performed on all turbid blood culture broths. Turbid
424 blood cultures with Gram-negative bacilli seen on microscopy also underwent
425 *B. pseudomallei* latex agglutination and immunofluorescence assay (IFA)
426 testing. AMD method was: 0.5ml of turbid blood culture broth dispensed into a
427 1.5ml Eppendorf tube, 1 drop of lysis buffer added and the suspension gently
428 mixed using a micropipette. 20µl of suspension was added to 3 drops of
429 chase buffer in a 0.6ml Eppendorf tube, and mixed by gentle pipetting. The
430 AMD test strip was then inserted and the result read after 15 minutes
431 according to manufacturer's instructions (Appendix 1). Latex agglutination
432 was performed as previously described (17): 5µl of latex reagent was mixed
433 with one drop of uncentrifuged blood culture broth on a glass slide, gently
434 rocked and observed for agglutination within 2 minutes. Positive (heat killed *B.*
435 *pseudomallei)* and negative (heat killed *B. thailandensis*) controls were
436 performed each day by mixing 5µl control with 5µl of latex reagent.
437 IFA, based on the previously described method (19), was performed as
438 follows: 10µl of turbid blood culture broth was spread on a glass slide to
439 create a thin smear and allowed to air dry. The slide was then fixed by
440 flooding with absolute methanol for 10 minutes at room temperature, and
441 again allowed to air dry. 10µl of IFA reagent (containing 5µg/ml monoclonal
442 antibody 4B11 and 20µg/ml Alexa Fluor® 488 conjugated-Goat Anti-Mouse
443 IgG) was then applied to the smear, covered with a coverslip and incubated at
444 room temperature for 5 minutes before observing under a fluorescent

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445 microscope (Nikon Eclipse E600 microscope with the U-FL Epifluorescence
446 attachment) at a magnification of x1000 using oil immersion. Periphery of
447 bacilli showing strong apple-green fluorescence was recorded as a positive
448 result (10). Positive results were semi-quantified using a scheme adapted
449 from the International Union against Tuberculosis and Lung Disease
450 (IUATLD) guidelines for quantification of Acid Fast Bacilli (AFB) (26) (Table
451 S3). A minimum of 100 fields were examined before slides were recorded as
452 IFA negative (i.e. no apple-green fluorescent bacilli seen). IFA was performed
453 on the same day that the smear was made whenever possible, and the next
454 day if not. Slides of heat-killed (1×10^6 CFU/ml in PBS at 80°C for 1 hour) *B.*
455 *pseudomallei* and *B. thailandensis* were used as positive and negative
456 controls, respectively, for each batch of immunofluorescence tests. Routine
457 laboratory staff performed the latex agglutination and AMD tests, whilst all IFA
458 tests were performed by LB blinded to the latex and AMD test results. All
459 blood culture work was performed in a Biosafety Cabinet at Laboratory
460 containment level 2 according to normal local practice.
461
462 *Pus, sputum, sterile fluid:* AMD and IFA were performed on all pus, sputum,
463 and sterile fluid samples on receipt in the laboratory. Latex agglutination is not
464 validated for these sample types and was therefore not performed on these
465 samples. IFA was performed as above using 1µl of sputum, pus or sterile
466 fluid sample to make the original smear. AMD was performed as follows: 20µl
467 of pus, sterile fluid or thick/viscous sputum samples was mixed with 3-4 drops
468 of lysis buffer by vortexing for 15 seconds. 20µl of this mixture was then
469 added to 3 drops of chase buffer in a 0.6ml Eppendorf tube and mixed by

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470 gentle pipetting. An AMD test strip was inserted and the result read after 15
471 minutes. Thin/watery sputum samples were processed in the same way
472 except that 50µl of sample was mixed with 2-3 drops of lysis buffer initially.
473 Routine laboratory staff performed AMD tests, while IFA was performed by LB
474 blinded to the results of AMD.
475
476 *Urine:* AMD was performed using 50µl of neat urine added to 3 drops of
477 chase buffer in a 0.6ml Eppendorf tube and mixed by gentle pipetting. AMD
478 test strip was then inserted and the result read after 15 minutes. AMD tests
479 were performed by routine laboratory staff. If AMD on neat urine was negative
480 then urine concentration was performed when concentrators (Minicon B15,
481 Merck Millipore Ltd) were available. 5ml of urine was concentrated x100 using
482 these simple table-top concentrators according to manufacturer's instructions.
483 AMD was then performed as above using 20µl of concentrated urine. Neither
484 latex agglutination nor IFA have been validated for use directly with urine
485 samples and were therefore not performed on these samples.
486
487 *Sera:* Admission sera (stored at -80°C) from culture-confirmed melioidosis
488 cases were retrieved and AMD performed as follows: 35µl serum added to the
489 AMD test strip followed by 3 drops of Chase buffer. Results were read after 15
490 minutes as previously.
491
492 Each new box of AMD test strips underwent quality assurance by testing one
493 strip from the box as follows: a single colony of *B. pseudomallei* positive
494 control (Lao clinical isolate UI 8976) was emulsified, using a sterile loop, in 2

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495 drops of lysis buffer. 3 drops of chase buffer were then added to this bacterial
496 suspension and mixed gently by pipetting before the AMD test strip was
497 inserted and read as previously described.
498
499 *Data Analysis*
500 Diagnostic sensitivity and specificity of the AMD, latex agglutination and IFA
501 on the different sample types were calculated using culture as the reference
502 standard. The sensitivity and specificity of AMD was compared with latex
503 agglutination and IFA on turbid blood cultures containing Gram-negative
504 bacilli, and with IFA alone for pus, sputum, and sterile fluid samples using a
505 two-sample test of proportions. In patients confirmed with melioidosis (by
506 culture from any sample) whose urine was culture negative for *B.*
507 *pseudomallei* a two-sided Fisher's exact test was used to analyze the
508 association between urine AMD positivity and 1) disseminated vs. localized
509 melioidosis, and 2) presence or absence of *B. pseudomallei* bacteraemia.
510 Melioidosis cases were defined as 'disseminated' when *B. pseudomallei*
511 bacteraemia was present and/or there was clinical/ radiological or
512 microbiological evidence of multiple sites of disease. Cases were defined as
513 'localized' if only one site of disease was present e.g. pneumonia or parotitis.
514 Analysis was done using STATA, v14.2 (College Station, TX, USA).

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

536 Conflict of interests: DA declares that InBios has licensed the mAb 4C4,
537 produced by the AuCoin laboratory, from the University of Nevada, Reno.
538 There are no other conflicts of interest to declare.

539

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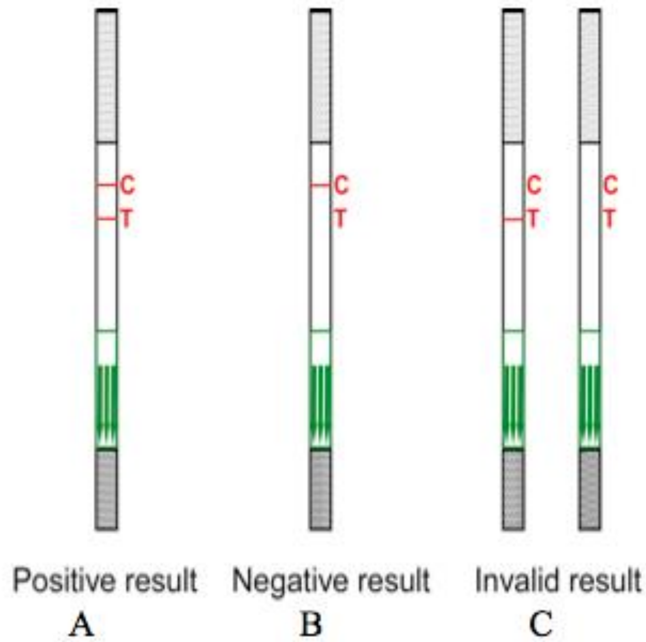
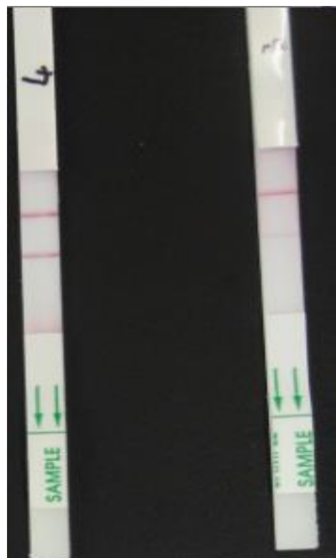
540 **Appendix 1. Interpretation of AMD reactivity according to**
541 **manufacturer's (InBios) instructions:**



542

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



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655 **Figure Legends**

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

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

660 Figure 1b. Sample Flow diagram: Pus samples

661 Figure 1c. Sample Flow diagram: Sputa samples

662 Figure 1d. Sample Flow diagram: Sterile Fluid samples

663 Figure 1e. Sample Flow diagram: Urine samples

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

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

667 Figure 1f. Sample Flow diagram: Serum samples from culture-confirmed
668 melioidosis cases diagnosed 26th June – 18th December 2014

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670 **Tables**

671 Table 1. Diagnostic sensitivity and specificity for all tests and sample types
672 compared with culture as the reference standard.

Specimen type (n)	**Test** (n)	**Sensitivity %** (95% Confidence Interval)	**Specificity %** (95% Confidence Interval)
Turbid Blood culture with GNB (252)	AMD (252)	99.0 (94.6-100%)	100 (97.6-100%)
	Latex (237)	99.0 (94.5 – 100)	100 (97.4 – 100)
	IFA (176)	100 (94.8 – 100)	98.1 (93.4 – 99.8)
	p-value	NS*	NS**
Pus (139)	AMD (139)	47.1 (23.0 - 72.2)	100 (97.0-100)
	IFA (95)	66.7 (29.9 – 92.5)	90.7 (82.5-95.9)
	p-value	0.338	0.0006
Sputum (23)	AMD (23)	33.3 (0.84 – 90.6)	100 (83.2-100)
	IFA (23)	100 (29.2 – 100)	85.0 (62.1-96.8)
	p-value	0.083	0.072
Sterile Fluid (46)	AMD (46)	0 (0 – 84.2%)	100 (92.0 – 100)
	IFA (43)	100 (15.8 – 100)	100 (91.4-100)

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	p-value	0.046	1.0
Urine Selected (241)	AMD (241)	86.7 (59.5 – 98.3%)	90.7 (86.2-94.2%)

673 NS = not significant; * p = 0.994 for AMD v latex, 0.402 for Latex v IFA, 0.405 for IFA
674 v AMD; ** p = 1.0 for AMD v latex, 0.107 for Latex v IFA, 0.169 for IFA v AMD
675
676

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677 Table 2. Urine AMD results according to site of disease in melioidosis cases
678 who were urine culture-negative for *B. pseudomallei* (n = 93*).

Site of disease (n)		**Urine AMD result**	
		Positive	Negative
Disseminated (61)	Bacteraemic (49)	15	34
	Not bacteraemic (12)	3	9
Localized (32)		3	29

679 *1 patient did not have sufficient data available to categories site of disease

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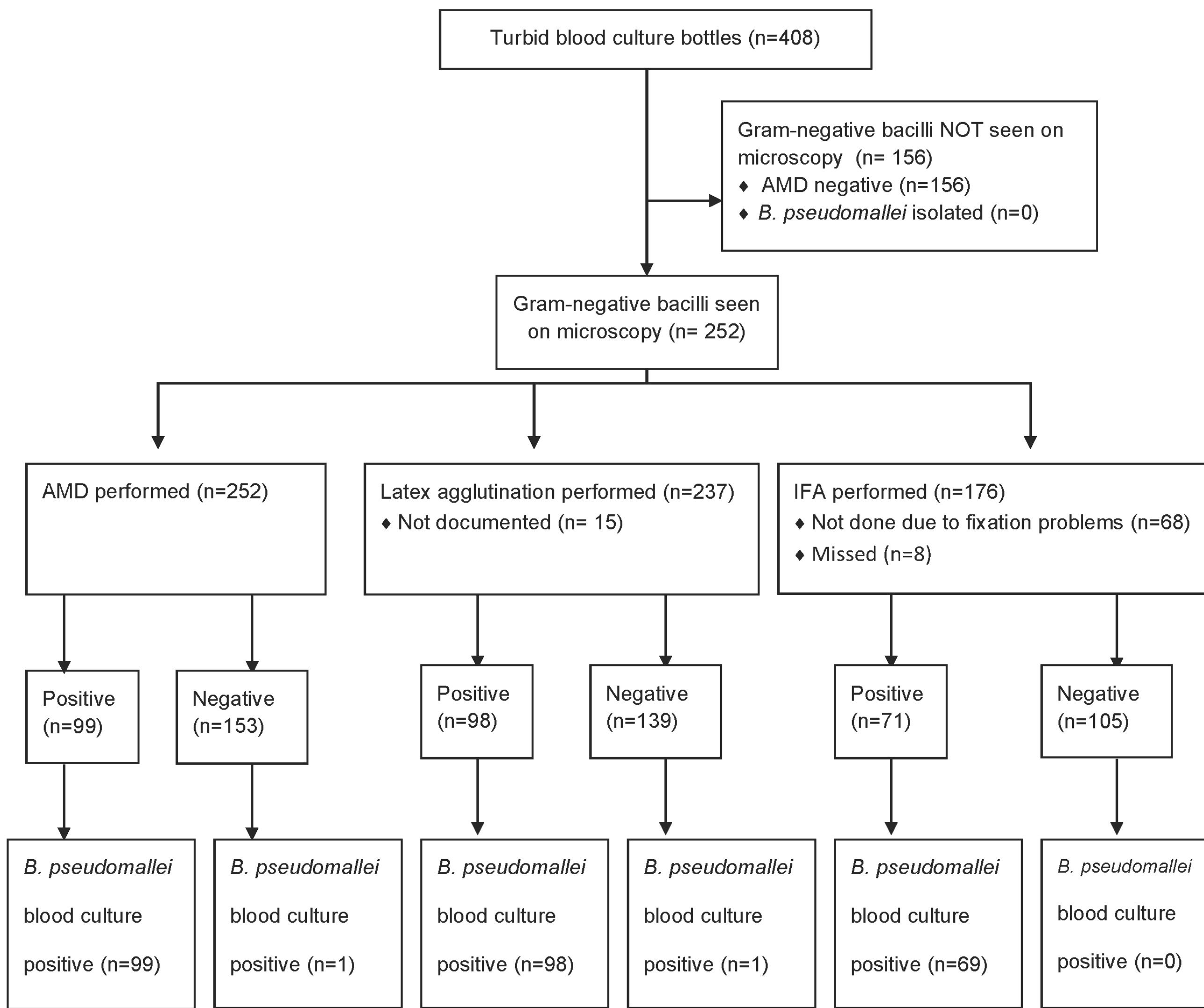

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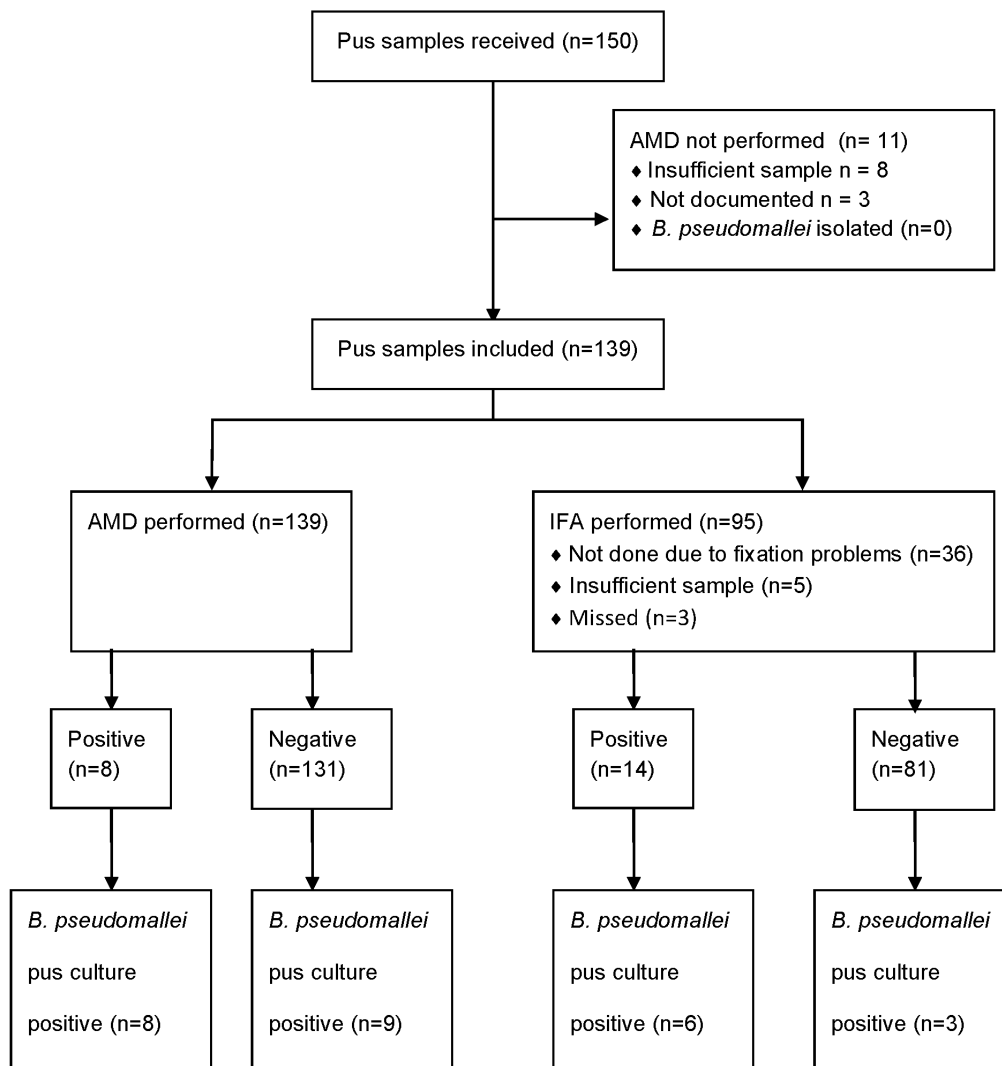

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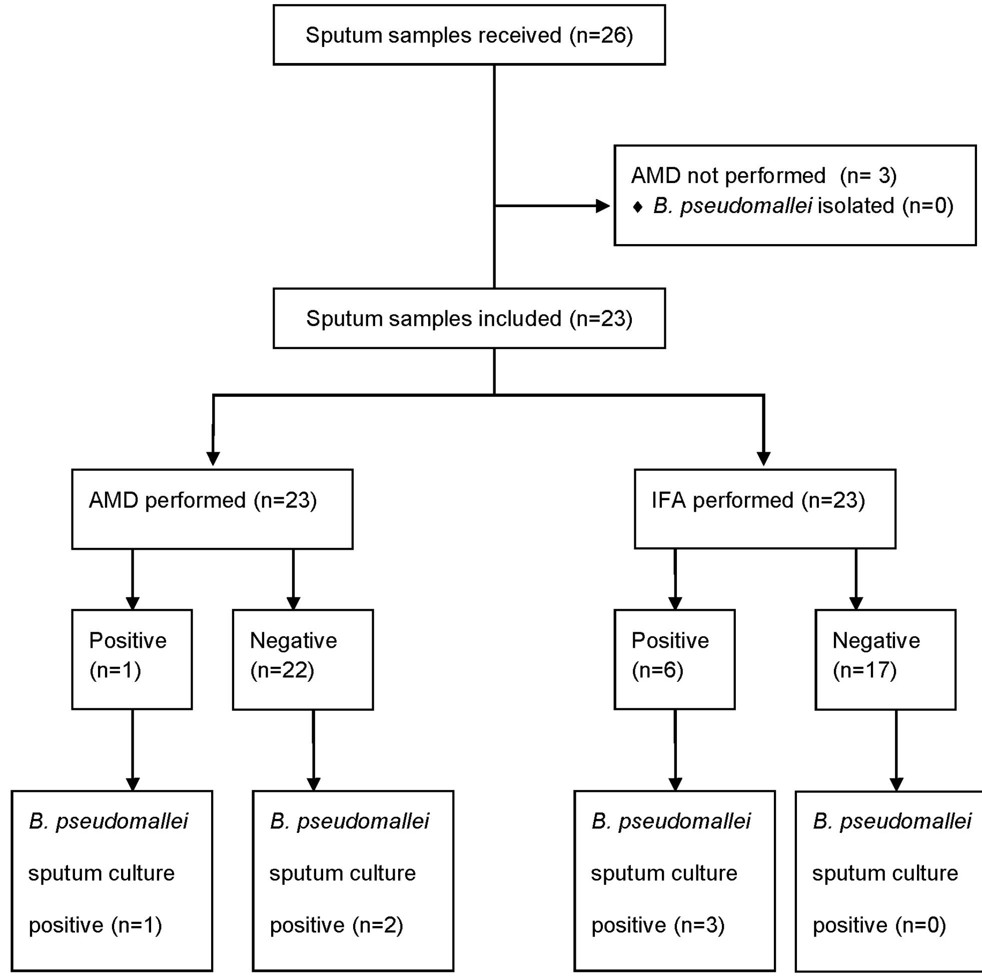

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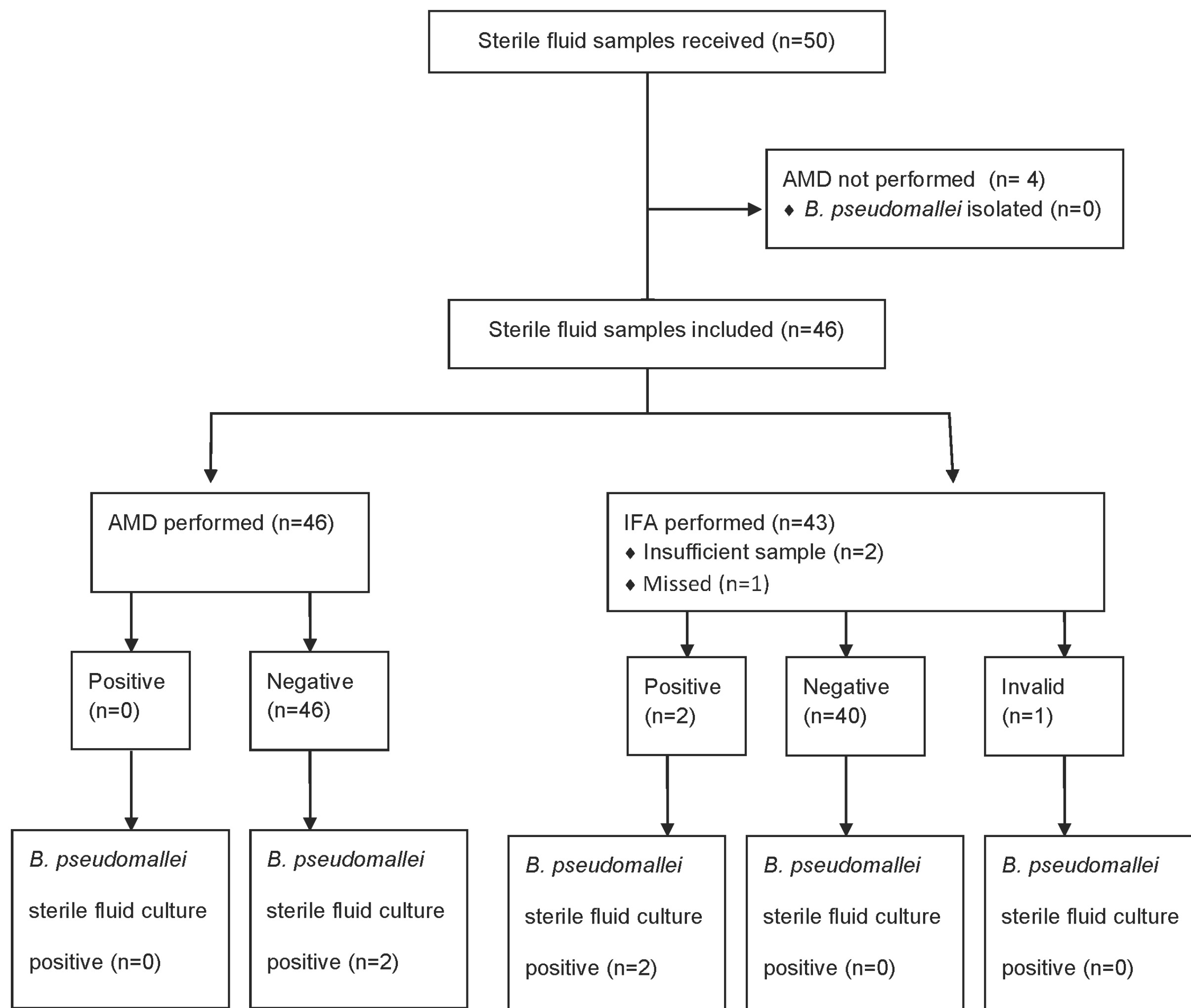

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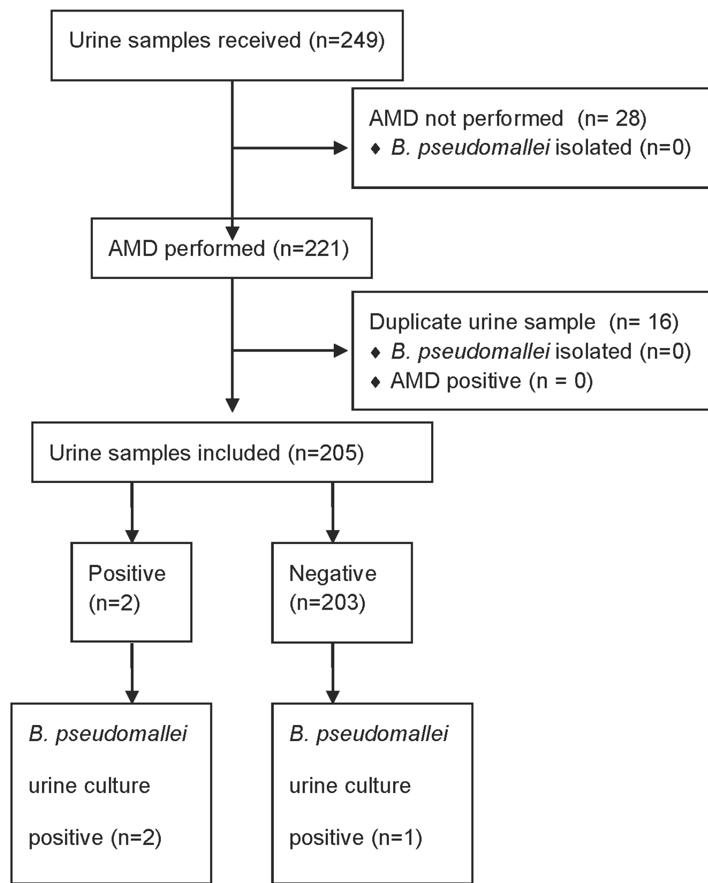

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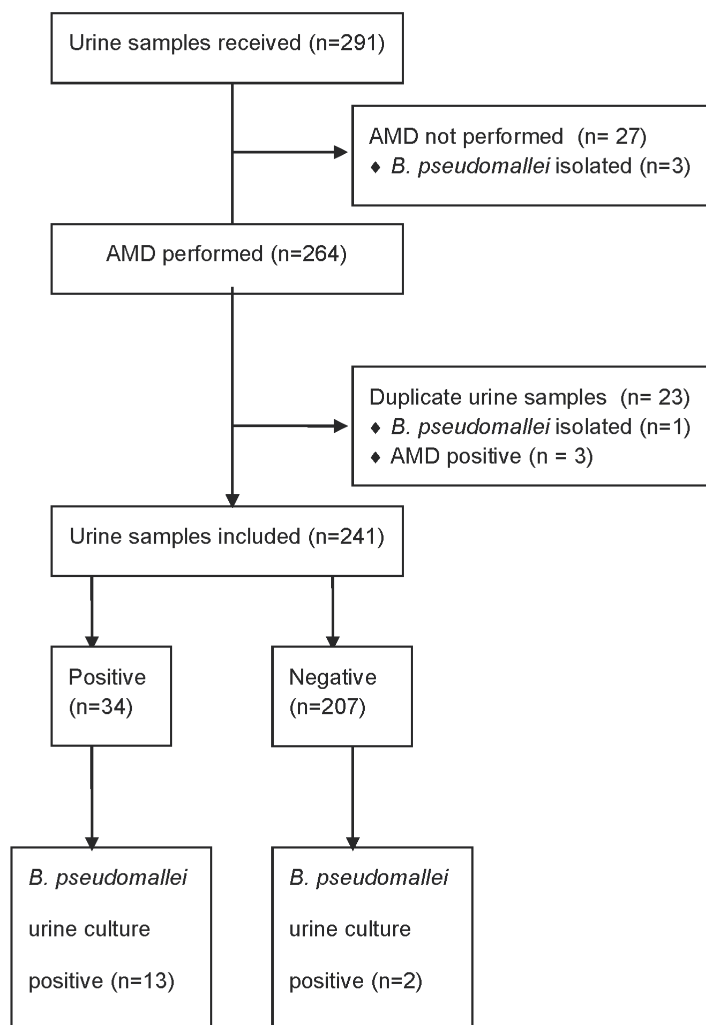

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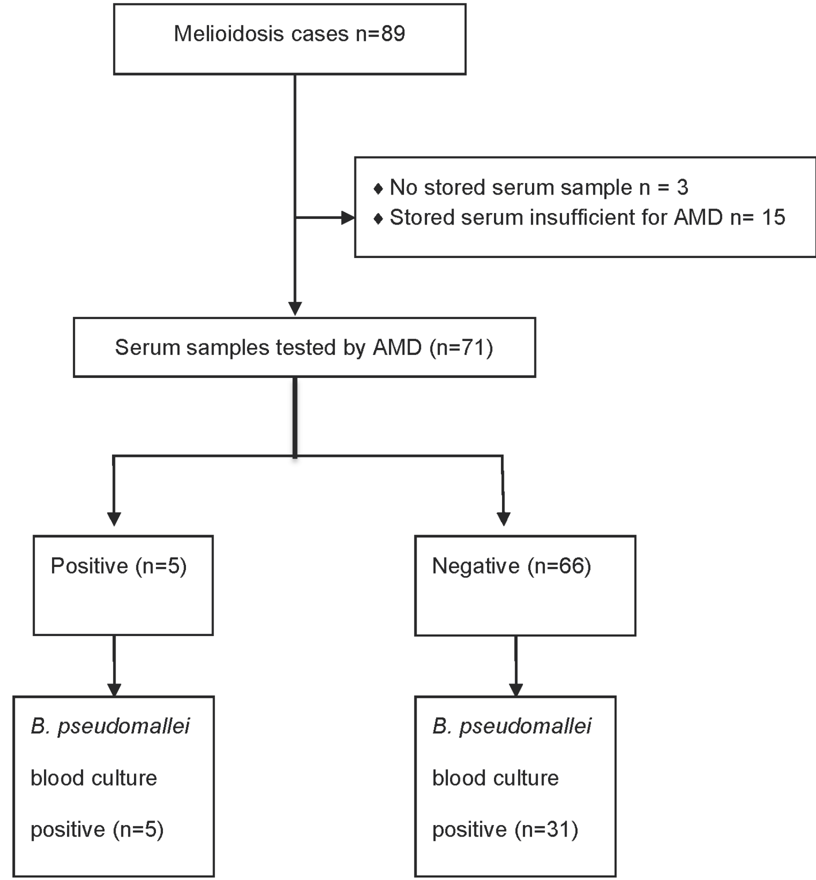

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