1 **Benchmarking CRISPR-BP34 for point-of-care melioidosis detection in LMIC: a molecular**
2 **diagnostics study**
3
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Summary

Background

Melioidosis is a grossly neglected but often-fatal tropical disease. The disease is named “a great mimicker” after its broad clinical manifestations, which makes disease diagnosis challenging and time-consuming. To improve diagnosis, we developed and evaluated the performance of the CRISPR-Cas12a system called “CRISPR-BP34” to detect *Burkholderia pseudomallei* DNA across clinical specimens from patients suspected to have melioidosis.

Methods

We conducted a prospective, observational study to document time taken for diagnosis, antibiotics prescribed during the waiting period, and follow up 28-day mortality outcomes in 876 culture-confirmed melioidosis admitted or referred to a hospital in northeast Thailand between 1 October 2019 and 31 December 2022. In the last six months, we performed CRISPR-BP34 detection on clinical specimens (blood, urine, respiratory secretion, pus and other body fluids) collected from 330 patients with suspected melioidosis and compared its performance to the current gold-standard culture-based method. Discordant results were validated by three independent qPCR tests.

Findings

A window of 3-4 days was required for the gold-standard culture diagnosis, which resulted in delayed treatment. 199 [22·7%] of 876 patients died prior to diagnosis results while 114 [26·3%] of 433 follow-up cases had been diagnosed, treated, but died within 28 days of admission. An application of CRISPR-BP34 reduced the average sample-to-diagnosis time to 1·1 day for blood sample, 2·3 hours for urine, and 3·3 hours for respiratory secretion, pus and other body fluids. We demonstrated an improved sensitivity of CRISPR-BP34 (106 [93·0%] of 114 positive cases, 95% CI 86·6 - 96·9) compared to the culture approach (76 [66·7%] of 114 positive cases, 95% CI 57·2 - 75·2); while maintaining similar specificity (209 [96·8%] of 216 negative cases, 95% CI 93·4-98·7) to the culture (216 [100 %] of 216 negative cases, 95% CI 98·3-100·0).

Interpretation

The sensitivity, specificity, speed, and window of clinical intervention offered by the CRISPR-BP34 support its prospective use as a point-of-care diagnostic tool for melioidosis with future development focused on scalability and cost reduction.

Funding

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71 **Research in context**

72 **Evidence before this study**

73 Melioidosis is an often-severe infectious disease caused by the bacterium *Burkholderia*
74 *pseudomallei*. It is estimated to affect 165,000 individuals annually worldwide, of which 89,000
75 cases are fatal. The disease diagnosis is challenging due to its diverse clinical presentations, low
76 awareness, limited diagnostic options, or even a lack of diagnostic tests. A PubMed search
77 conducted from the database inception to 17 October 2023, using the terms "melioidosis" AND
78 "diagnosis test," yielded 210 results, 40 of which presented clinical evaluations of rapid
79 melioidosis diagnostic tests. Antigen-based diagnostic tests, which detect the presence of *B.*
80 *pseudomallei*, reported high specificity (median = 98·6%, IQR 94·0 - 100·0), but low sensitivity
81 (median = 57·1%, IQR = 44·3 - 82·5). The test sensitivity suffers from the often-low concentration
82 of the bacterial antigens in patients' samples, which can vary by specimen type and stage of
83 infection. Antibody-based diagnostic tests that detect host antibodies against *B. pseudomallei*
84 typically exhibit satisfactory specificity (median = 94·5%, IQR = 88·6 - 96·2) but poor sensitivity
85 (median = 80·2%, IQR = 71·0 - 88·1). These tests are often impacted by variations in antibody
86 responses to *B. pseudomallei* and the duration required for antibody production. Furthermore,
87 standardisation remains challenging due to the influence of different serum titres on sensitivity and
88 background of the tests. Likewise, quantitative PCR exhibits a high degree of specificity (median
89 = 99·8%, IQR = 91·6-100·0), but an observed low sensitivity for melioidosis (median = 77·1%,
90 IQR = 20·8-97·8), which is likely attributed to the low concentration of initial bacterium or the
91 genetic heterogeneity of *B. pseudomallei* genomes or both. Additionally, these studies consistently
92 reported a demand for improved speed and ease of implementation in resource-limited settings
93 where melioidosis is endemic. With the limitations of current diagnostic methods, a culture-
94 confirmed approach with 60% sensitivity, 100% specificity, and a diagnosis time of 2-7 days still
95 stands as the gold standard for melioidosis diagnosis.

96 **Added value of this study**

97 To date, no study has measured the impact of delayed diagnosis on melioidosis. We assessed the
98 number of deaths occurring prior to culture-confirmed diagnosis and those after diagnosis but
99 within 28 days post-admission, highlighting the urgent need for prompt action. To address this, we
100 developed the CRISPR-BP34 diagnostic test, which utilises isothermal amplification of *B.*
101 *pseudomallei* DNA followed by site-specific detection using a CRISPR-associated Cas12a

102 enzyme. We successfully implemented this assay in a resource-limited setting in northeast
103 Thailand, where the disease prevalence is among the highest in the world. The assay achieved a
104 diagnostic sensitivity and specificity of 93·0% and 96·8%, respectively, with an estimated limit of
105 detection (LoD) ranging from 50-250 cfu/mL. Based on the specimen type, early diagnosis can be
106 achieved within four hours, or one day after patient admission. This is significantly faster than the
107 gold-standard test that typically takes several days. Furthermore, the ultra-sensitive CRISPR-BP34
108 assay detected low *B. pseudomallei* levels in hemoculture bottles, which could be missed due to
109 mixed infections, aseptic issues, or other causes. This contribution could play a vital role in
110 preventing undiagnosed melioidosis.

111 **Implications of all available evidence**

112 The CRISPR-BP34 assay holds potential for the management and control of melioidosis. Its speed
113 and heightened sensitivity enable early diagnosis and treatment, which are crucial for saving
114 patients' lives. Additionally, the minimal setup and user-friendly learning curve make the assay
115 ideal for resource-limited settings.

116 **INTRODUCTION**

117 Melioidosis is a neglected tropical disease with a high case-fatality (10-50%) even when
118 appropriately treated[1]. The global disease burden expressed in disability-adjusted life-years
119 (DALYs) is 4·64 million, 99% of which is accounted for by years of life lost (YLL)[2]. High
120 mortality may be explained by the disease disproportionately affecting rural populations in low-
121 and middle-income countries (LMICs)[3], where poor socioeconomic conditions often result in
122 patients seeking healthcare when the disease has reached a terminal or critical phase[4]. Melioidosis
123 is caused by *Burkholderia pseudomallei*, an environmental bacterium in soil and water across the
124 tropical regions of Asia Pacific[5], South[6] and Southeast Asia[7]. However, the disease remains largely
125 underreported due to its non-specific clinical manifestations that can "mimic" several other
126 diseases. A lack of disease awareness in clinics and communities together with the paucity of
127 diagnostic facilities leads to missed or delayed diagnosis. With early diagnosis and appropriate
128 treatment, the case fatality rate from melioidosis can be decreased to 9% as observed in Australia[8].
129

130 Clinical specimens from patients with suspected melioidosis are typically screened for the
131 presence of *B. pseudomallei* using microbial culture, which has been the gold-standard diagnostic
132 method for the last three decades. This method is imperfect, with a specificity of 100% but a
133 sensitivity of 60%[9]. *B. pseudomallei* exhibits slower growth in the laboratory conditions compared
134 with other pathogens[10]. This delay can lead to the proliferation of other bacteria or fungi within
135 the sample due to mixed infection and/or contamination, and results in failure to detect *B.*
136 *pseudomallei*. When cultured successfully, *B. pseudomallei* colonies can be mistaken for
137 environmental contaminants, necessitating correct identification by a skilled microbiologist.
138 Moreover, the combined time required for both growing and identifying *B. pseudomallei* could
139 extend to 7 days, resulting in inevitably delays in disease diagnosis[9]. Culture-free antigen-based
140 and nucleic acid-based tests such as a lateral flow immunoassay (LFI)[11], an immunofluorescence
141 assay (IFA)[12], polymerase chain reaction (PCR)[13–15], or 16S rRNA sequencing[16] have been
142 developed for diagnosing melioidosis. However, the variability in bacterial concentrations[17,18]
143 across clinical specimens leads to a limited sensitivity of 58·2% (95% CI, 34·1 – 78·9%) for LFI[19]
144 and 63·8% (45·6 – 78·7%) for IFA[19], whilst tests that offer higher sensitivity require thermal
145 cyclers or sequencing machines[16], which are rarely available in rural settings.
146

147 We hypothesised that an improved sensitivity and specificity through the detection of *B.*
148 *pseudomallei* DNA from direct clinical specimens can improve melioidosis diagnosis. One such
149 method is clustered regularly interspaced short palindromic repeats (CRISPR)-based diagnostics,
150 which involves amplifying the pathogen's DNA using isothermal recombinase polymerase
151 amplification (RPA) and then utilising the sequence-specific recognition of CRISPR-Cas
152 endoribonuclease at the DNA target. This approach has been applied to other bacterial pathogens
153 including *Mycobacterium tuberculosis*[20] and has been demonstrated to improve diagnosis and
154 treatment responses. We previously described a robust CRISPR-Cas12a-based detection of
155 genomic DNA of *B. pseudomallei in vitro*[21]. Here, we addressed the issues surrounding delayed
156 diagnosis of melioidosis, established a diagnostic protocol for our recently developed CRISPR-
157 Cas12a system[21] (hereafter termed CRISPR-BP34), and determined its sensitivity and specificity
158 for diagnostic uses.
159
160 **METHODS**
161 **Study design and participants**
162 Two related studies were conducted and reported here. Study 1 assessed the time required for
163 melioidosis diagnosis through culture and patient outcomes, while Study 2 evaluated the diagnostic
164 performance of the CRISPR-BP34 assay (figure 1).
165
166 Study 1 was a prospective observational cohort study of melioidosis patients at Sunpasitthiprasong
167 Hospital, a tertiary care hospital in Ubon Ratchathani, Thailand, between 1 October 2019 and 31
168 December 2022 (figure 1A; appendix pp 4-7). Participants were eligible for inclusion if they had
169 cultured-confirmed infection of *B. pseudomallei* from any clinical samples. Participants with
170 tuberculosis, human immunodeficiency virus (HIV), or immunosuppressive conditions that might
171 impact the infectious outcomes were excluded. One of the study objectives were to assess the time
172 taken for diagnosis, treatments administered, and the resulting infection outcomes. The study
173 received ethical approval from the Sunpasitthiprasong Hospital Ethical Review Board (015/62C)
174 and the Oxford Tropical Research Ethics Committee (OxTREC, 25-19). This study is registered
175 with Thai Clinical Trial Registry (TCTR20190322003). All participants provided written informed
176 consent.
177

178 Study 2 was a diagnostic evaluation of the sensitivity and specificity of the CRISPR-BP34
179 prototype assay[21] conducted at Sunpasitthiprasong Hospital between 26 May 2022 and 31
180 December 2022 (figure 1B). Participants were identified through the hospital computer system and
181 were included if they were suspected of having melioidosis and had sufficient leftover clinical
182 samples. The study received ethical approval from the Ethical Review Board of Sunpasitthiprasong
183 Hospital (029/65C) and received ethical exemption from Chiang Mai University (9190/2565).
184
185 **Procedures**
186 For Study 1, all patients with culture-confirmed melioidosis were identified through the hospital
187 computer system. Consents were obtained to collect the clinical records including the diagnosis
188 duration, the antibiotics prescribed before the culture confirmation (appendix pp 6-7), and the
189 patients' 28-day survival status from admission, which was tracked through telephone follow-ups.
190 Data also included patient demographics, symptoms, the interval between symptom onset and
191 seeking healthcare, the duration of culture-confirmed diagnosis, and the antibiotics prescribed
192 during the unconfirmed period. In routine clinical practice, blood, urine, respiratory
193 secretions/fluid (sputum, tracheal suction, and pleural fluid), and other available body fluid/tissues
194 (pus, limb tissue and synovial fluid), were consecutively collected for culture from patients
195 suspected of having melioidosis. The standard culture methods used for each specimen type are
196 outlined in the appendix (p 6), serving as a reference for evaluating CRISPR-BP34 in Study 2. One
197 millilitre of leftover sample from the culture was obtained from the hospital microbiology
198 laboratory and stored at -20 °C for CRISPR-BP34 screening. Once the culture results arrived, a
199 head-to-head CRISPR-BP34 assay was performed (appendix pp 9-11, 13-19).
200
201 Different types of clinical samples require sample-specific preparation due to the variable amounts
202 of target bacterial cells[17], host cells and inhibitors present in each sample. Further details of how
203 the assay was performed on each sample type is described in the appendix (pp 9-11). Briefly, for
204 each clinical sample, human cells were first depleted using a simple buffer system to selectively
205 lysed human cells, leaving a pellet of bacterial cells. Bacterial genomic DNA was then extracted
206 from the pellet using either hot alkaline lysis or a GeneJet spin column (ThermoFisher Scientific,
207 Waltham, Massachusetts, USA, #K0721), depending on the pellet size. *B. pseudomallei* DNA was
208 amplified in an RPA reaction (TwistDX, Maidenhead, Berkshire, UK, #TABAS03KIT), and the

209 resulting amplicons were added into a 50-µL CRISPR reaction, comprising of CRISPR RNA
210 (crBP34), LbCas12a protein and FAM-biotin probes (IDT, Singapore) (appendix p 8). This
211 reaction was incubated at 37 °C for 60 minutes, after which a HybriDetect lateral flow dipstick
212 (Milenia Biotec, Giessen, Germany, #MGHD1) was directly immersed into the reaction and
213 allowed to develop for 5 minutes before reading by eye. A positive result was defined as the
214 appearance of an upper band (anti-IgG) on the dipstick. The assay was performed in a batch of 10
215 samples with each batch consisting of culture positive and negative samples to avoid batch effect.
216 For all batches, *B. pseudomallei* positive sample and distilled water were also used as positive and
217 negative controls, respectively. The sample-to-result time was recorded. The CRISPR-BP34
218 results were interpreted by three different readers who were blinded to the patient disease status
219 and culture results.
220
221 Discordant results between culture and the CRISPR assay were tested by quantitative PCR (qPCR)
222 using three primer sets listed in the appendix (pp 11, 25-26). Given a large discrepancy in reported
223 diagnostic sensitivity of PCR primers[19] and a high sequence diversity of *B. pseudomallei*, the use
224 of primer combinations ensured an increased coverage of the detection. The qPCR cycle threshold
225 (ct) values were recorded and used as a proxy for bacterial loads.
226
227 To estimate the potential range of the limit of detection (LoD) of the CRISPR-BP34 assay, we also
228 conducted *in vitro* experiments by inoculating genetically modified *Escherichia coli* that
229 harboured a target DNA of the CRISPR-BP34 in its genome (appendix p 8) into blood and urine
230 samples from a single healthy donor at different concentration ranging from 0, 10, 50, 100, 250,
231 500, 2500 and 5000 cfu/mL. Two to five biological replicates were performed for each experiment.
232 CRISPR-BP34 detection was performed on blood and urine as described in the appendix (pp 9-
233 11). Blood and urine were selected to represent the most common types of clinical specimens
234 processed from patients with suspected melioidosis. We cautioned the caveats over the use of
235 modified *E. coli* as a surrogate for *B. pseudomallei* (appendix p 9) which was a necessary choice
236 to obviate laboratory safety concerns.
237
238 **Outcomes**

239 The outcome of study 1 was 28-day mortality and factors associated with mortality in melioidosis
240 patients with culture-confirmed diagnosis. The outcome of study 2 was the evaluation of CRISPR-
241 BP34's clinical sensitivity, specificity, and assay time against the gold-standard culture-confirmed
242 diagnosis.
243

244 **Statistical analysis**

245 The number of melioidosis cases admitted to Sunpasitthiprasong Hospital during the study period
246 determined the sample size for study 1, while the minimal sample size for study 2 was determined
247 using the formula[22] $n = z^2 p(1-p) /d^2$ where "z" is the 95% confidence interval at 1·96; "p" is the
248 prevalence at 0·5; and "d" represents the margin of error at 0·1. At least 96 melioidosis and 96
249 non-melioidosis patients were required for the CRISPR-BP34 diagnostic test. Patient demographic
250 data were summarised using medians, interquartile ranges (IQR), and proportions. To evaluate 28-
251 day mortality associated with each factor, Kaplan-Meier survival curves, alongside univariable
252 and multivariable Cox proportional hazard regression were employed with hazard ratios (HR) and
253 their 95% confidence interval reported. Factors associated with melioidosis mortality were chosen
254 from literature reviews[1,2]. These include patient demographics (age, sex and self-reported
255 ethnicity), symptoms (presence or absence of specific symptoms), time from symptom onset to
256 primary healthcare and diagnosis (categorised as ≤ 7 days, 8 - 14 days, 15 - 21 days, or > 21 days),
257 and antibiotic prescription (presence or absence). Sensitivity and specificity were separately
258 calculated for culture and CRISPR-BP34, categorised by sample type (blood; urine; respiratory
259 secretion; and pus, tissue and other body fluids), and by total specimens. If a patient had multiple
260 samples, only the earliest sample was used for these calculations. Exact 95% confidence interval
261 was estimated using binomial assumption. McNemar's test was used to compare the performance
262 of culture and CRISPR assay using paired data. For comparison of variables observed from the
263 assays with non-parametric distribution, Wilcoxon signed-rank tests were used. All tests were two-
264 sided, with a significance level of 0·05. R (version 4.3.1) was employed for all analyses and data
265 visualisation.
266

267 **Role of the funding source**

268 The funders of the study had no role in study design, data collection, data analysis, data
269 interpretation, or writing of the report.

270

271 **RESULTS**

272 **Time to diagnosis, empirical antibiotic treatment, and outcomes**

273 Of 876 culture-confirmed melioidosis patients admitted or referred to Sunpasitthiprasong Hospital
274 during October 2019 to December 2022, 199 died before the culture results arrived (figure 1A).
275 Of 433 patients who survived to culture results and were enrolled in this study, the infectious
276 outcomes were known for 431 patients. Among these, 114 patients died within 28 days after first
277 presentation to Sunpasitthiprasong Hospital. The minimum fatality of 313 [35·7%] of 876 patients
278 based on the combined data is consistent with 35% mortality reported in Thailand[22]. On average,
279 patients lived 65 km (IQR 40 – 100 km) from the hospital, with the majority working as the
280 agricultural workers (table 1). Melioidosis patients displayed diverse clinical manifestations with
281 persistent fever being the most common symptom (table 1; appendix pp 5, 23). Patients
282 experienced symptoms for a median of seven days (IQR 3 -14 days) before seeking medical care
283 at the local or central healthcare centres, which subsequently referred them to Sunpasitthiprasong
284 Hospital within one day (IQR 0 – 4 days). Multiple samples were collected from patients for
285 culture-based diagnosis upon their initial admission or referral and during their stay at
286 Sunpasitthiprasong Hospital as clinically indicated. The median duration between the first sample
287 collection and the first positive culture result was four days (IQR 3 - 5 days), at which point
288 melioidosis diagnosis was confirmed. Time from symptom onset to diagnosis averaged 16 days
289 (IQR 9 – 27 days), shorter in patients who died (12 days, IQR 7 – 19 days) compared to survivors
290 (18 days, IQR 10 – 31 days; table 1; appendix p 20), emphasising the need for prompt clinical
291 intervention to avert fatal outcomes.

292

293 In Thailand, patients suspected of melioidosis are recommended to receive empiric treatment with
294 intravenous ceftazidime or carbapenem antibiotics, such as meropenem or imipenem, for initial
295 intensive monotherapy[23]. Antibiotic administration data was available for 433 patients of which
296 49 (11·3%) and 54 (12·5%) respectively received ceftazidime or carbapenem monotherapy upon
297 their first presentation to Sunpasitthiprasong Hospital (figure 2A; table 1; appendix p 20). The
298 remainders received other treatments including a monotherapy or combinations of antibiotics
299 known to be ineffective in treating melioidosis such as lincosamides[24], macrolides, penicillin with
300 first- and second-generation cephalosporin[25-27], and ineffective third-generation cephalosporins

301 such as ceftriaxone[28] – an inevitable practice to cover the broad spectrum of infection when the
302 causative agents are unknown (figure 2B, 2C). A lower mortality (5 [10·2%] of 49) was observed
303 in patients who received ceftazidime monotherapy than in patients who received other types of
304 treatments (109 [28·5%] of 382; Cox regression p-value 0·0026; HR 0·32 (95% CI 0·13 - 0·79;
305 appendix pp 27-28). No other treatments or factors displayed significant association with 28-day
306 mortality in our studied population in the multivariable Cox regression analysis. To increase the
307 use of ceftazidime monotherapy from 11·3% to 100% at the patient's initial presentation, more
308 rapid diagnosis is necessary.
309
310 **Clinical evaluation of the CRISPR-BP34 assay**
311 We developed the CRISPR-BP34 assay to detect *B. pseudomallei* DNA, which demonstrated high
312 sensitivity and specificity *in vitro*[21]. We conducted spiking experiments to determine the limit of
313 detection of the CRISPR-BP34 in blood and urine samples, estimating it to be 250 cfu/mL and 50
314 cfu/mL, respectively (figure 3A, 3B). To determine whether CRISPR-BP34 test is sufficiently
315 sensitive to detect the bacterium across various clinical samples, we enumerated the numbers of
316 *B. pseudomallei* recovered from diverse specimen types (figure 3C). Our results showed that the
317 number of *B. pseudomallei* present in most common specimens such as urine (median = $2{\cdot}6 \times 10^4$
318 cfu/mL), sputum (median = $8{\cdot}8 \times 10^7$ cfu/mL), pus and other body fluids (median = $5{\cdot}4 \times 10^7$
319 cfu/mL) were greater than the CRISPR-BP34's limit of detection. Since direct blood samples had
320 a lower *B. pseudomallei* concentration (median = 1·5 cfu/mL) than the limit of detection, we
321 substituted direct blood samples with hemoculture positive samples (median = $7{\cdot}3 \times 10^7$ cfu/mL)
322 to ensure sufficient bacterial concentration. Hemoculture positive samples are blood samples
323 cultured to enhance bacterial growth, but the bacterial identity remains unknown (figure 3D).
324
325 To test the hypothesis that CRISPR-BP34 assay would be more sensitive and faster than culture-
326 confirmed approach, we enrolled and collected clinical samples from 114 melioidosis and 216
327 non-melioidosis patients (figure 1B). Of these, 54 melioidosis cases were also enrolled in study 1.
328 Specifically, 20 melioidosis and 12 non-melioidosis patients had samples collected across multiple
329 specimen types (figure 4A), while 94 melioidosis and 204 non-melioidosis patients had a single
330 sample type collected (figure 4B). Using first sample available from each patient, we estimated
331 the overall diagnostic sensitivity and specificity of both methods. Our findings showed an overall

332 sensitivity of CRISPR-BP34 of 93·0% (106 of 114 samples, 95% CI 86·6 – 96·9), higher than the
333 sensitivity of culture at 66·7% (76 of 114 samples, 95% CI 57·2 - 75·2) (figure 4C; appendix p
334 29). The overall specificity of the CRISPR-BP34 was 96·8% (209 of 216 samples, 95% CI 93·4 -
335 98·7), compared to 100% (216 of 216 samples, 95% CI 98·3 - 100·0) for culture (figure 4D; table
336 2). Sensitivity of CRISPR-BP34 versus culture for individual sample types was generally higher
337 for CRISPR-BP34, as follows: hemoculture at 100% (41 of 41 samples) vs 58·5% (24 of 41
338 samples); urine at 70·4% (19 of 27 samples) vs 51·9% (14 of 27 samples); respiratory secretions
339 and fluids at 94·2% (49 of 52 samples) vs 84·6% (44 of 52 samples); and other body fluids and
340 tissue at 100% (15 of 15 samples) vs 73·3% (11 of 15 samples) (figure 4C; table 2). Specificity
341 of CRISPR-BP34 versus culture for individual sample types was equivalent or slightly lower for
342 CRISPR-BP34, as follows: hemoculture at 94·6% (70 of 74 samples) vs 100% (74 of 74 samples);
343 urine at 98·6% (70 of 71 samples) vs 100% (71 of 71 samples); respiratory secretions and fluids
344 at 98·1% (53 of 54 samples) vs 100% (54 of 54 samples); and other body fluid and tissue at 96·7%
345 (29 of 30 samples) vs 100% (30 of 30 samples) in CRISPR-BP34 and culture assay, respectively.
346 A McNemar's test further confirmed different performance of the CRISPR-BP34 test and culture
347 for total specimens (p-value < 0.0001; appendix p 29).
348
349 In addition, the CRISPR-BP34 assay provided faster results and significantly reduced the
350 turnaround time for all sample types compared to culture approach (figure 4E, Wilcoxon test p-
351 value < 0.0001 for all sample types). For culture positive samples, the median sample-to-result
352 time was 2·5 days (IQR 1·8 – 3·3 days) for hemoculture, and 3·9 days (IQR 3·7 – 4·1 days) for
353 urine, respiratory secretion, and fluids, as well as other body fluids and tissues. In contrast, the
354 average sample-to-result time for the positive CRISPR-BP34 assay was 1·1 days (IQR 0·7 – 1·5
355 days) for blood required hemoculture, 2·3 hours (IQR 2·3 – 2·4 hours) for urine, and 3·3 hours
356 (IQR 3·1 – 3·4 hours) for respiratory secretion, and fluids, as well as other body fluids and tissues.
357

358 **Potential use case**

359 Among 114 melioidosis patients, 20 had various sample types and multiple samples collected over
360 time for each type (figure 4A; appendix p 21), providing an opportunity to investigate how each
361 patient was diagnosed and treated in real-world scenarios. Early specimens were collected on the
362 first or within a few days of admission or referral to aid disease diagnosis, while late specimens

were taken at 3-, 5- or 7-days intervals following antibiotic prescription to assess response to the treatment (note that late specimens were not included in this study's analysis). A proportion of early specimens collected from patients later confirmed to have melioidosis yielded negative culture results. This could be attributed to either the concentration of *B. pseudomallei* falling below the culture's limit of detection or the samples being contaminated with other fast-growing bacterial species (appendix p 21). One striking example was a hemoculture bottle from a melioidosis case that was contaminated with coagulase-negative staphylococci, a skin commensal (appendix p 21). The CRISPR-BP34 assay, however, reported *B. pseudomallei* positive from this contaminated blood bottle, consistent with subsequent qPCR experiments confirming the presence of *B. pseudomallei* DNA and with the patient's final diagnosis. We observed a high cycle threshold (ct) score of *B. pseudomallei* in the contaminated hemoculture bottle and other contaminated samples (median = 32·3, IQR 30·5 - 35·9), compared to average ct values detected in *B. pseudomallei* positive hemoculture (median = 14·0, IQR 13·4 - 14·9; appendix p 22). This likely indicates that the *B. pseudomallei* population was outcompeted by contaminant species. Cross-contamination incidents like this are not uncommon in laboratories with limited resources such as in rural Thailand, which could result in an underestimation of the true incidence of melioidosis[29]. Regardless, for all cases being followed, CRISPR-BP34 provided earlier identification of *B. pseudomallei* than the culture method (appendix p 21).

DISCUSSION

Our study revealed that the gold-standard culture diagnosis required a 3–4-day window, with observed delayed treatment and patient deaths before and after culture diagnosis. Implementing CRISPR-BP34 could potentially reduce sample-to-diagnosis time to approximately a day for blood samples, and less than four hours for urine, respiratory secretions, pus, and other body fluids. CRISPR-BP34 displayed greater sensitivity while maintaining a comparable level of specificity. However, our study has limitations. Notably, severely ill patients died before we could reach them, thereby leading to number of deaths being underestimated. Nevertheless, our findings echo the problems[2] of delayed disease diagnosis and imperfect treatment during uncertain diagnoses, both of which may independently or collectively contribute to fatalities.

393 To improve the appropriate and timeous initiation of melioidosis treatment, we developed a
394 CRISPR-BP34 assay (appendix pp 9-19), tested the sample-type specific protocols (figure 3D),
395 and evaluated the test performance (figure 4). To our knowledge, the estimated LoD of the assay
396 at the range of 50 – 250 cfu/mL is the lowest among reported melioidosis rapid diagnosis tests
397 without requiring extensive equipment such as a qPCR machine or a UV microscope. Although
398 CRISPR-BP34 could detect *B. pseudomallei* at the concentration as low as 50 cfu/mL, the
399 miniscule volume of specimens utilised by the CRISPR approach means that the test can be skewed
400 by inaccurate pipetting or handling errors. Thus, for direct blood samples, we recommend using
401 CRISPR-BP34 on DNA extracted from hemoculture (enriched media) instead of direct blood
402 samples to maintain high sensitivity. For common clinical specimens with high bacterial loads
403 (over 10^3 cfu/mL) such as hemoculture samples, genitourinary fluids, respiratory secretions, as
404 well as pus and other body fluids, CRISPR-BP34 could be used directly on DNA extracted from
405 these samples. Consequently, CRISPR-BP34 exhibited high level of sensitivity of 93·0%
406 compared to 66·7% sensitivity for overall samples of culture approach (figure 4C).
407
408 We observed a slight drop in the CRISPR test specificity being 96·8% compared to 100%
409 specificity of culture approach across all sample types (figure 4D). Some of the “false” positives
410 could be “true” but “missed” diagnosis cases as result of current imperfect diagnosis techniques
411 including culture-confirmed approach[9] and qPCR[19] with suboptimal primers. Certain false
412 positives might also arise when high copy numbers of genetic materials or RPA amplicons were
413 mixed or handled in a confined bench setting, which is sometimes unavoidable in crowded space
414 of resource-limited laboratories. Alternatively, the CRISPR-BP34 complex might unintentionally
415 target other DNA sequences, resulting in false positives. However, the latter is likely mitigated by
416 the double-layered specificity provided by RPA primers and CRISPR RNA; each of which were
417 carefully designed using the genomic database of over 40,000 bacterial and human DNA[21]. To
418 ensure robust test, suggestions to minimise the DNA cross-contamination which could generate
419 false positives were documented in the appendix (p 11).
420
421 While our findings support the potential of CRISPR-BP34 as point-of-care diagnostic tool, further
422 technical refinement is needed to enhance user-friendliness, scalability, and cost-effectiveness.
423 Ongoing efforts are directed toward aligning CRISPR-BP34 with WHO’s point-of-care

424 diagnostics guideline[30] (appendix pp 12 and 30). CRISPR-BP34's implementation has the
425 potential to facilitate prompt initiation of life-saving treatment (appendix p 21) and has garnered
426 positively feedback Ministry of Public Health Thailand and regional health authorities. We believe
427 that a robust melioidosis rapid test, designed for resource-constraint settings, could also prove
428 effective in resource-rich environments.

429

430 **Contributors**

431 CChe and SPW conceived the study and secured funding. PC, SS, CU, HT, CChe and SPW,
432 designed the study. SP, PB, KA, NA, YD, AF, GW, PA, PK, VW, and CCho were involved in
433 study implementation under CChe and SPW supervision. CChe and SPW did the analysis. CChe,
434 SPW, PC, SJP, NPJD, NRT, and CU interpreted the data. SP, SPW, and CChe verified underlying
435 data of the study. All authors had full access to all the data in the study and had final responsibility
436 for the decision to submit for publication. CChe and SPW wrote the first draft. All authors read
437 and approved the manuscript.

438

439 **Data sharing**

440 Deidentified participant data that underlie the results reported in the article will be made available
441 upon request. Proposals should be directed to the corresponding author, claire@tropmedres.ac.
442 Proposals will be reviewed based on compliance with the informed consent and scientific merit.

443

444 **Declaration of interests**

445 All authors declare no competing interests.

446

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455

456 **Figure legends**

457 **Figure 1: Study design:** (A) A diagram summarises the mortality of melioidosis patients who
458 were admitted or referred to Sunpasitthiprasong Hospital between October 2019 and December
459 2022. All cases were identified by culture-confirmed diagnosis, which commonly arrived on day
460 4. A substantial number of patients died prior to the arrival of the gold-standard culture test result.
461 (B) A diagram presents our case-control study to evaluate the use of CRISPR-BP34 assay as an
462 alternative rapid diagnostic test. Specimens were frozen upon arrival to enable subsequent
463 screening with the CRISPR-BP34 test and head-to-head comparison with the culture method.
464 Asterisk marks the utility of hemoculture, a procedure to enrich bacterium recovered from patient
465 blood.

466

467 **Figure 2: Burden of delayed diagnosis on melioidosis treatments and outcomes:** (A) An UpSet
468 plot summarises the antibiotic prescription when patients first presented to Sunpasitthiprasong
469 Hospital, either as a monotherapy or combination therapy. (B) A histogram presents the number
470 of patients who remained undiagnosed at a daily interval after their admission or referral to
471 Sunpasitthiprasong Hospital from October 2019 to December 2022. Black arrow denotes the date
472 when a culture-confirmed melioidosis result was available for most patients. (C) A histogram
473 summarises choices of antibiotics prescribed to patients who remained undiagnosed for
474 melioidosis each day after their admission or referral date. Prescriptions of ceftazidime or
475 carbapenem drugs either as monotherapy or combination therapy are colored in blue, with black
476 arrow marking the first day admission.

477

478 **Figure 3: Limit of detection of the CRISPR-BP34 test and its proposed diagnostic pipeline**
479 (A and B) Lateral flow dipsticks present the limit of the CRISPR-BP34 test in blood and urine
480 samples, respectively, which are the two most common specimen types. The dipsticks are shown
481 as two biological replicates, each exhibiting estimated LoD of 50 cfu/mL for urine and 250 cfu/mL
482 for blood. (C) A chart summarises the number of *Burkholderia pseudomallei* recovered from
483 different specimen types. Green bar indicates the range of the CRISPR test's LoD. (D) A drawing
484 illustrates an overview of the CRISPR-BP34 diagnostic pipeline based on clinical samples
485 including blood, genitourinary fluid, respiratory secretion, pus and other body fluid that were

486 routinely collected from patients suspected of melioidosis. Bacterial pathogens in blood are
487 typically enriched for growth to the detectable threshold (250 cfu/mL) through hemoculture
488 process.
489

490 **Figure 4 Sensitivity and specificity of the culture and the CRISPR approaches**
491 (A) Combined panels represent combined panels of diagnostic results from 32 patients with
492 various specimen types. Top to bottom panels summarise results from hemoculture, genitourinary
493 fluid, respiratory secretion, and pus or other body fluids, respectively. For patients with multiple
494 samples per each specimen type, only the first sample is presented. Rows in each panel display
495 results from different diagnostic tests including the gold-standard culture, IFA, latex agglutination,
496 and the CRISPR-BP34 test (top to bottom). Each column corresponds to data from each patient
497 with disease status marked as melioidosis (black) or non-melioidosis (grey). (B) Findings from
498 298 patients with a single specimen type are summarised and presented in the same order as in
499 (A). The sensitivity (C), specificity (D) and sample-to-diagnosis time (E) of the gold-standard are
500 compared with those of CRISPR-BP34 assay. Dots indicate the actual values, with the 95%
501 confidence intervals represented by dashed lines (culture) and solid lines (CRISPR-BP34).
502

503 **References**

504 1 Wiersinga WJ, Virk HS, Torres AG, *et al.* Melioidosis. *Nat Rev Dis Primers* 2018; **4**: 17107.

505 2 Birnie E, Virk HS, Savelkoel J, *et al.* Global burden of melioidosis in 2015: a systematic
506 review and data synthesis. *The Lancet Infectious Diseases* 2019; **19**: 892–902.

507 3 Limmathurotsakul D, Golding N, Dance DAB, *et al.* Predicted global distribution of
508 *Burkholderia pseudomallei* and burden of melioidosis. *Nat Microbiol* 2016; **1**: 15008.

509 4 Savelkoel J, Dance DAB, Currie BJ, Limmathurotsakul D, Wiersinga WJ. A call to action:
510 time to recognise melioidosis as a neglected tropical disease. *The Lancet Infectious Diseases*
511 2022; **22**: e176–82.

512 5 Currie BJ, Mayo M, Ward LM, *et al.* The Darwin Prospective Melioidosis Study: a 30-year
513 prospective, observational investigation. *The Lancet Infectious Diseases* 2021; **21**: 1737–46.

514 6 Mohapatra PR, Mishra B. Burden of melioidosis in India and South Asia: Challenges and
515 ways forward. *The Lancet Regional Health - Southeast Asia* 2022; **2**: 100004.

516 7 Chantratita N, Phunpang R, Yarasai A, *et al.* Characteristics and one year outcomes of
517 melioidosis patients in Northeastern Thailand: A prospective, multicenter cohort study. *The*
518 *Lancet Regional Health - Southeast Asia* 2023; **9**: 100118.

519 8 Stewart JD, Smith S, Binotto E, McBride WJ, Currie BJ, Hanson J. The epidemiology and
520 clinical features of melioidosis in Far North Queensland: Implications for patient
521 management. *PLoS Negl Trop Dis* 2017; **11**: e0005411.

522 9 Limmathurotsakul D, Jamsen K, Arayawichanont A, *et al.* Defining the True Sensitivity of
523 Culture for the Diagnosis of Melioidosis Using Bayesian Latent Class Models. *PLoS ONE*
524 2010; **5**: e12485.

525 10 Peeters M, Ombelet S, Chung P, *et al.* Slow growth of *Burkholderia pseudomallei* compared
526 to other pathogens in an adapted blood culture system in Phnom Penh, Cambodia. *Journal of*
527 *Medical Microbiology* 2019; **68**: 1159–66.

528 11 Houghton RL, Reed DE, Hubbard MA, *et al.* Development of a Prototype Lateral Flow
529 Immunoassay (LFI) for the Rapid Diagnosis of Melioidosis. *PLoS Negl Trop Dis* 2014; **8**:
530 e2727.

531 12 Tandhavanant S, Wongsuvan G, Wuthiekanun V, *et al.* Monoclonal Antibody-Based
532 Immunofluorescence Microscopy for the Rapid Identification of *Burkholderia pseudomallei*
533 in Clinical Specimens. *The American Journal of Tropical Medicine and Hygiene* 2013; **89**:
534 165–8.

535 13 Lowe W, March JK, Bunnell AJ, O'Neill KL, Robison RA. PCR-based Methodologies Used
536 to Detect and Differentiate the *Burkholderia pseudomallei* complex: *B. pseudomallei*, *B.*
537 *mallei*, and *B. thailandensis*. *Curr Issues Mol Biol* 2014; **16**: 23–54.

538 14 Price EP, Dale JL, Cook JM, *et al.* Development and Validation of *Burkholderia*
539 *pseudomallei*-Specific Real-Time PCR Assays for Clinical, Environmental or Forensic
540 Detection Applications. *PLoS ONE* 2012; **7**: e37723.

541 15 Novak RT, Glass MB, Gee JE, *et al.* Development and Evaluation of a Real-Time PCR Assay
542 Targeting the Type III Secretion System of *Burkholderia pseudomallei*. *J Clin Microbiol*
543 2006; **44**: 85–90.

544 16 Gee JE, Sacchi CT, Glass MB, *et al.* Use of 16S rRNA Gene Sequencing for Rapid
545 Identification and Differentiation of *Burkholderia pseudomallei* and *B. mallei*. *J Clin*
546 *Microbiol* 2003; **41**: 4647–54.

547 17 Wuthiekanun V, Limmathurotsakul D, Wongsuvan G, *et al.* Quantitation of *B. Pseudomallei*
548 in clinical samples. *Am J Trop Med Hyg* 2007; **77**: 812–3.

549 18 Wongsuvan G, Limmathurotsakul D, Wannapasni S, Chierakul W, Teerawattanasook N,
550 Wuthiekanun V. Lack of correlation of *Burkholderia pseudomallei* quantities in blood, urine,
551 sputum and pus. *Southeast Asian J Trop Med Public Health* 2009; **40**: 781–4.

552 19 Fairley L, Smith S, Maisrikrod S, Henning L. Systematic review and meta-analysis of
553 diagnostic tests for diagnosis of melioidosis. *Acta Trop* 2021; **214**: 105784.

554 20 Huang Z, LaCourse SM, Kay AW, *et al.* CRISPR detection of circulating cell-free
555 *Mycobacterium tuberculosis* DNA in adults and children, including children with HIV: a
556 molecular diagnostics study. *The Lancet Microbe* 2022; **3**: e482–92.

557 21 Wongpalee SP, Thananchai H, Chewapreecha C, *et al.* Highly specific and sensitive detection
558 of *Burkholderia pseudomallei* genomic DNA by CRISPR-Cas12a. *PLoS Negl Trop Dis* 2022;
559 **16**: e0010659.

560 22 Daniel WW. Biostatistics: A foundation for analysis in the health sciences., 7th edn. Hoboken:
561 John Wiley & Sons, 1999.

562 23 Hinjoy S, Hantrakun V, Kongyu S, *et al.* Melioidosis in Thailand: Present and Future.
563 *TropicalMed* 2018; **3**: 38.

564 24 Dance D. Treatment and prophylaxis of melioidosis. *Int J Antimicrob Agents* 2014; **43**: 310–8.

565 25 Karunakaran R, Puthucheary SD. *Burkholderia pseudomallei*: In vitro susceptibility to some
566 new and old antimicrobials. *Scandinavian Journal of Infectious Diseases* 2007; **39**: 858–61.

567 26 Hii SYF, Tandhavanant S, Phunpang R, *et al.* Antibiotic Susceptibility of Clinical
568 *Burkholderia pseudomallei* Isolates in Northeast Thailand from 2015 to 2018 and the
569 Genomic Characterization of *β* -Lactam-Resistant Isolates. *Antimicrob Agents Chemother*
570 2021; **65**: e02230-20.

571 27 Jenney AWJ, Lum G, Fisher DA, Currie BJ. Antibiotic susceptibility of *Burkholderia*
572 *pseudomallei* from tropical northern Australia and implications for therapy of melioidosis.
573 *International Journal of Antimicrobial Agents* 2001; **17**: 109–13.

574 28 Chaowagul W, Simpson AJ, Suputtamongkol Y, White NJ. Empirical cephalosporin treatment
575 of melioidosis. *Clin Infect Dis* 1999; **28**: 1328.

576 29 Rhodes J, Jorakate P, Makprasert S, *et al.* Population-based bloodstream infection surveillance
577 in rural Thailand, 2007–2014. *BMC Public Health* 2019; **19**: 521.

578 30 Land KJ, Boeras DI, Chen X-S, Ramsay AR, Peeling RW. REASSURED diagnostics to
579 inform disease control strategies, strengthen health systems and improve patient outcomes.
580 *Nat Microbiol* 2019; **4**: 46–54.