Adapting Microarray Gene Expression Signatures for Early Melioidosis Diagnosis

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ABSTRACT Melioidosis is caused by Burkholderia pseudomallei and is predominantly seen in tropical regions. The clinical signs and symptoms of the disease are nonspecific and often result in misdiagnosis, failure of treatment, and poor clinical outcome. Septicemia with septic shock is the most common cause of death, with mortality rates above 40%. Bacterial culture is the gold standard for diagnosis, but it has low sensitivity and takes days to produce definitive results. Early laboratory diagnosis can help guide physicians to provide treatment specific to B. pseudomallei. In our study, we adapted host gene expression signatures obtained from microarray data of B. pseudomallei-infected cases to develop a real-time PCR diagnostic test using two differentially expressed genes, AIM2 (absent in melanoma 2) and FAM26F (family with sequence similarity 26, member F). We tested blood from 33 patients with B. pseudomallei infections and 29 patients with other bacterial infections to validate the test and determine cutoff values for use in a cascading diagnostic algorithm. Differentiation of septicemic melioidosis from other sepsis cases had a sensitivity of 82%, specificity of 93%, and negative and positive predictive values (NPV and PPV) of 82% and 93%, respectively. Separation of cases likely to be melioidosis from those unlikely to be melioidosis in nonbacteremic situations showed a sensitivity of 40%, specificity of 54%, and NPV and PPV of 44% and 50%, respectively. We suggest that our AIM2 and FAM26F expression combination algorithm could be beneficial for early melioidosis diagnosis, offering a result within 24 h of admission.

KEYWORDS Burkholderia, diagnostics, gene expression, melioidosis, sepsis

Melioidosis is an infectious disease caused by Burkholderia pseudomallei, which commonly occurs in Southeast Asia, Northern Australia, and other tropical regions of Asia, South and Central America, and Africa (1). Melioidosis rates Northeast Thailand are increasing year by year, and in 2010 the incidence was 12.7 cases per 100,000 people (2). Signs and symptoms of the disease are nonspecific, which frequently leads to misdiagnosis and failure of treatment (3), resulting in poor clinical outcomes (1, 4–6). Septicemia with septic shock is the most common cause of death in melioidosis patients, with mortality rates above 40% (2, 7).

The gold standard for melioidosis diagnosis is bacterial culture, which has low sensitivity and takes several days to produce a definitive result (8, 9). Many approaches have been attempted to overcome these issues. Serologically based diagnostic methods have been developed based on detection of antibody against B. pseudomallei components. The indirect hemagglutinin assay (IHA) is routinely applied in hospitals to screen for melioidosis; however, it has been shown to lack sensitivity (69.5%) and
specificity (67.6%) in areas where *B. pseudomallei* is highly endemic (10–12). Moreover, the positive predictive value (PPV) of the IHA is very low (40.9%), making it unsuitable for melioidosis diagnosis (13). An enzyme-linked immunosorbent assay (ELISA) that detects antibodies against O-polysaccharide and hemolysin coregulated protein of *B. pseudomallei* had 72 to 83% sensitivity and >95% specificity (14, 15).

Latex agglutination using beads conjugated with *B. pseudomallei*-specific monoclonal antibodies could identify bacterial colonies isolated from clinical samples at a high sensitivity (99.1%) with a reduced time to report (16). An immunochromatography assay using capsule polysaccharide-specific monoclonal antibody to detect bacterial components had reasonable specificity (93.6%) but low sensitivity (85.7%) (17, 18), particularly when applied to blood samples (31.3% sensitivity) (19). PCR detection of the T3SS gene cluster provides >80% sensitivity (20), but direct detection of bacteria in clinical specimens is problematic, as pathogen concentrations are generally very low (21). A microarray approach using 20 recombinant and purified *B. pseudomallei* proteins to detect both short-term and long-term antibodies from melioidosis patient sera provided >80% sensitivity and >90% specificity, but this may not be an affordable diagnostic approach (22).

Previous studies have used microarray gene expression profiling to define signatures that differentiate melioidosis cases from other bacterial septicemias (23, 24). AIM2 (absent in melanoma 2) and FAM26F (family with sequence similarity 26, member F) are two genes that have been shown to be differentially expressed in septicemic melioidosis, other bacterial infections, and healthy controls (24). Implementation of microarrays as a diagnostic tool in a hospital setting is not practical; therefore, we used PCR assays to validate the diagnostic usefulness of these gene signatures by comparing (i) cases of bacterial culture-confirmed septicemic melioidosis to sepsis cases caused by other organisms and to healthy controls and (ii) nonbacteremic cases that were likely to be *B. pseudomallei* infections to nonbacteremic cases that were unlikely to be melioidosis.

**MATERIALS AND METHODS**

**Patient enrollment and sample selection.** This study was approved by the Nakhon Phanom Provincial Ethics Committee in Human Research, Thailand (IEC-NKP1 no. 20/2015) and by the Khon Kaen University Ethics Committee for Human Research and the Khon Kaen Hospital Ethics Committee (approval no. HE470506). A total of 879 patients with systemic inflammatory response syndrome (SIRS) admitted to the Nakhon Phanom Hospital were enrolled, and whole blood was collected. In addition, some enrollments also had plasma (n = 25) or buffy coat (n = 7) specimens prepared. Healthy controls were whole blood collected from healthy blood donors at the Nakhon Phanom Hospital. Written informed consent was obtained from all subjects. The enrollments were divided into bacteremic and nonbacteremic groups by hemoculture result. RNA from the bacteremic group was used to independently evaluate the candidate markers in training and validation groups selected chronologically. From the nonbacteremic groups by hemoculture result. RNA from the bacteremic group was used to independently evaluate the candidate markers in training and validation groups selected chronologically. From the nonbacteremic groups by hemoculture result. RNA from the bacteremic group was used to independently evaluate the candidate markers in training and validation groups selected chronologically.

Whole blood was collected for gene expression profiling and hemoculture. For RNA, 0.5 ml was mixed with 1 ml Tempus blood RNA buffer (Thermo Fisher Scientific, USA) and stored at −80°C until the day of analysis. Hemoculture was performed using 10 ml of blood added to a Bactec Plus Aerobic/F bottle and incubated in the Bactec FX blood culture system (BD Diagnostic Systems, USA) following the manufacturer’s instructions. Bottles were reported as negative if no signal was recorded up to 5 days. Bottles which signaled positive were subcultured onto sheep blood, chocolate, and MacConkey agar plates. Bacterial identification was performed by biochemical testing using analytical profile index kits (API20E; bioMérieux, France). The final diagnosis in the clinician’s record was used to confirm sepsis patients infected with non-*B. pseudomallei* bacteria (the “other sepsis” group), including one case that presented as “coagulase-negative staphylococcus” and was confirmed by the final diagnosis to be staphylococcus septicemia.

**8. pseudomallei latex agglutination.** Bottles that signaled positive were subcultured onto sheep blood agar and incubated for 18 to 24 h at 35°C. Single colonies from sheep blood plates growing Gram-negative bacilli were mixed with 10 μl of latex agglutination reagent (Mahidol University, Bangkok, Thailand) (25, 26) on a glass slide. Agglutination was detected visually after rotation for 2 min. A positive control (killed *B. pseudomallei*) and a negative control (killed *Burkholderia thailandensis*) were included on each day the test was used.

**B. pseudomallei**
Nonbacteremic classification. Nonbacteremic enrollment patients were categorized according to the outcome of antibiotic treatment into (i) a "likely to be melioidosis" group and (ii) an "unlikely to be melioidosis" group. The criteria used to classify each group were modified from a previous description and are shown in Table 1 (27).

Candidate gene selection. Candidate genes were selected from Illumina Sentrix BeadChip microarray transcriptomic profile data derived from comparing sepsis patients to healthy controls (24). AIM2 and FAM26F were selected as they best-distinguished septicemic melioidosis patients from other bacterial infections and healthy controls (see Fig. S1 in the supplemental material).

Gene expression quantification by real-time PCR and determination of cutoff values for melioidosis diagnosis. Total RNA was prepared using Tempus Spin RNA isolation kits (Thermo Fisher Scientific, USA) following the manufacturer’s instructions (the protocol was modified for an elution volume of 40 μl), and cDNA was synthesized using the ImProm-II reverse transcription system (Promega, USA) per the manufacturer’s directions. Real-time PCR was performed with the one-component hot start reaction mix FastStart Essential DNA Green mastermix (Roche Diagnostics GmbH, Germany) on a

![FIG 1 Patient enrollment design and classification. Suspected sepsis patients from Nakhon Phanom Hospital (n = 879) were enrolled in the study. Using hemoculture results, the enrollments were divided into bacteremic and nonbacteremic groups. The bacteremic group (n = 62) was assessed for the ability of AIM2 and FAM26F expression to differentiate septicemic melioidosis-positive cases from sepsis cases caused by other organisms in two independent sample sets, the training (n = 25) and validation (n = 37) sets. In the nonbacteremic group (n = 817), cases that showed either no improvement in symptoms with antibiotic treatment (n = 166) or improvement in symptoms without antibiotic treatment (n = 23) were excluded. Fifteen of the cases that showed improved outcomes after treatment with cefazidime and/or carbapenem and/or amoxicillin (n = 266) were classified as "likely to be melioidosis" based on the following additional supporting criteria: either the presence of multiple liver and/or splenic abscess(es) on abdominal ultrasound or of pneumonia on chest X-ray. Thirteen of the cases that showed improved outcomes after treatment with any antibiotic except cefazidime and/or carbapenem and/or amoxicillin (n = 362) were selected as "unlikely to be melioidosis." Of note, two melioidosis cases in the nonbacteremic group had a previous positive test for B. pseudomallei at another culture site within 30 days.

TABLE 1 Criteria used to classify cases likely and unlikely to be melioidosis among the nonbacteremic cases

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Likely to be melioidosis</td>
<td>Patient showed improved outcome after treatment with the generally used melioidosis antimicrobial regimen (ceftazidime and/or carbapenem and/or amoxicillin-clavulanate) and showed the presence of either multiple liver or splenic abscesses on ultrasound or pneumonia on chest X-ray</td>
</tr>
<tr>
<td>Unlikely to be melioidosis</td>
<td>Patient showed improved outcome after treatment with any antibiotic except those generally used in the melioidosis antimicrobial regimen (ceftazidime and/or carbapenem and/or amoxicillin-clavulanate)</td>
</tr>
</tbody>
</table>
LightCycler 96 instrument (Roche Diagnostic GmbH, Germany). Cycling conditions were as follows: (i) initial denaturation at 95°C for 5 min and (ii) 3-step 40-cycle PCR performed at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. GAPDH was used as a reference gene. Phytohemagglutinin was used as an internal positive control, and nucleic acid-free water was used as a negative nontemplate control. The primer sequences were as follows: GAPDH forward primer, 5′-TGAAGGATCTGAAGG, and reverse primer, 5′-GCATCGGGTGACAGATGTA (24). Cycle threshold (Ct) data of the target genes was normalized to GAPDH expression using the formula \( \Delta \Delta CT = (C_{T, \text{target}} - C_{T, \text{GAPDH}}) \), and the expression level was calculated using the 2\(^{-\Delta\Delta CT}\) method.

Hcp1-ELISA. The presence of hemolysin coregulated protein 1 (Hcp1) antibodies in plasma samples collected from culture-confirmed melioidosis patients in the training and validation sets \((n = 25)\) was evaluated by ELISA. Recombinant Hcp1 (Hcp1) antigen was prepared as previously described (14, 29). ELISA was performed using Hcp1 at 2.5 μg/ml with 1:2,000 dilution of horseradish peroxidase-conjugated rabbit anti-human IgG (15). The results were determined at an optical density at 450 nm (OD450), with OD cutoff values above 1.165 considered positive (14).

TTS1 real-time qPCR. DNA was extracted from seven buffy coat samples of culture-confirmed melioidosis patients in the training set using a QIAamp DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions and tested for the \(B.\) pseudomallei type three secretion system 1 (TTS1) gene. The protocol was modified for an elution volume of 40 μl. Real-time PCR analysis was performed on a Bio-Rad CFX platform (Bio-Rad, Hercules, CA). Samples were tested in duplicate with each reaction mixture containing 1 μl of total genomic DNA template. Peptidylprolyl isomerase A (PPIA; also called cyclophilin A) was used as an internal control. Each mixture consisted of a total volume of 10 μl with 1× iTaq SYBR green Supermix (Bio-Rad, Hercules, CA) and a 0.2 μM final concentration of forward and reverse primers. The primer sequences were as follows: BpTT4176 forward primer, 5′-CGTCTCTTATCTGTGCGACCAATG, and BpTT4290 reverse primer, 5′-CGTCACCGGTTAGTATC (30); PPIA_S primer, 5′-CGTGGGACAAACAAATGG, and PPIA_A primer, 5′-TTGCAAAACACACTGCT (31). The thermal cycling conditions were 95°C for 15 min, followed by 95°C for 15 s and 60°C for 30 s for 45 cycles. Positive controls were 200 pg \(B.\) pseudomallei K96243 DNA for the TTS1 gene and healthy whole-blood DNA for the PPIA gene.

**Data analysis.** Statistical analysis was performed using Prism software version 6 (GraphPad Software, USA) and SPSS version 17.0 (SPSS, Inc., USA). The normality of the data was determined by the D’Agostino and Pearson omnibus normality test. Statistical differences in normally distributed data were determined by Student’s \(t\) test, while nonnormally distributed data were analyzed by the Mann-Whitney test or by one-way analysis of variance (ANOVA) with the Kruskal-Wallis posttest. The significant differences in categorical data were evaluated by chi-square test. The post hoc power analysis for this experimental sample size was calculated at >90% power with a 95% confidence level.

**RESULTS**

Expression of \(AIM2\) and \(FAM26F\) was significantly upregulated in septicemic melioidosis patients compared to other sepsis cases and healthy controls. We screened 879 patients by hemoculture for septicemic bacterial infection (Fig. 1). Based on the bacterial growth results, samples were classified into bacteremic (62/879, 7%) and nonbacteremic sets (817/879, 93%).

We assessed our chosen candidate genes, \(AIM2\) and \(FAM26F\), for their ability to differentiate septicemic melioidosis from other bacterial sepsis cases and healthy controls by real-time PCR (Fig. 2). For the training set, we enrolled 30 cases, 12 of which were culture-confirmed melioidosis cases, 13 of which were culture confirmed to be other bacterial infections, and 5 of which were healthy control cases (Table S3). Both gene candidates could distinguish between melioidosis and other sepsis patients \((AIM2 P < 0.01; FAM26F P < 0.001)\), whereas differences between septicemic melioidosis and healthy controls could only be seen in \(AIM2 (P < 0.01)\). Neither gene candidate could differentiate other sepsis patients from healthy controls (Fig. 2). The validation set with 42 enrolled cases, which included 21 culture-confirmed melioidosis cases, 16 cases culture confirmed as other bacterial infections, and 5 healthy controls (Table S3), confirmed our findings from the training set (Fig. 2).

Combining the training and validation set data (72 cases) showed that the expression differences between melioidosis cases and other infections was highly significant for \(AIM2\) and \(FAM26F\) \((P < 0.0001)\). Similarly, there were significant differences in gene expression levels between the septicemic melioidosis cases and healthy controls \((AIM2 P < 0.0001 \text{ and } FAM26F P < 0.05)\) in the combined data set (Fig. 2).

The expression ratios of both \(AIM2\) and \(FAM26F\) showed significant differences between melioidosis cases (\(Burkholderia pseudomallei\)) and other infections (\(B.\) pseudomallei versus others, \(P < 0.0001\) for both genes) and between melioidosis cases and
healthy controls (B. pseudomallei versus healthy, \( P < 0.0001 \) for both genes). Only AIM2 showed a significant difference in expression ratio between other infections and healthy controls (others versus healthy, \( P < 0.05 \)) (see Fig. S2 in the supplemental material). PCR efficiencies, as measured by the slope of the AIM2, FAM26F, and GAPDH real-time curves, were 1.23, 1.11, and 1.82, respectively.

**Efficacy of AIM2 and FAM26F expression for diagnosis of melioidosis cases in bacterial culture-confirmed samples.** A receiver operating characteristic curve (ROC) analysis was used to establish the most appropriate cutoff values for AIM2 and FAM26F expression for melioidosis diagnosis (Fig. 3). Starting with 100% sensitivity, the highest specificities for AIM2 or FAM26F expression were low, at 62% and 46%, respectively. To increase the specificity, we looked for points on the ROC curve where sensitivity and specificity were both above 80%. The best expression cutoff value for AIM2 was 0.061 (83% sensitivity, 85% specificity, and 83% PPV), and for FAM26F, the best cutoff value was 0.015 (83% sensitivity, 92% specificity, and 91% PPV) (Table 2).

Next, we examined combining the AIM2 and FAM26F signatures to develop an algorithm to improve the efficacy of melioidosis diagnosis using the training set. Screening out cases with AIM2 expression values below 0.033 before using a FAM26F cutoff of 0.020 (Table 3) gave the best diagnosis results. These cutoff values showed 75% sensitivity, 100% specificity, 81% NPV, and 100% PPV in the training set (Table 3). The results from the validation set revealed 86% sensitivity, 88% specificity, 82% NPV, and 90% PPV. For the combined data set, our melioidosis diagnostic algorithm showed 82% sensitivity, 93% specificity, 82% NPV, and 93% PPV (Table 3).

We compared our combination AIM2 and FAM26F algorithm with Hcp1-ELISA and TTS1-PCR methods for melioidosis diagnosis. Our gene combination algorithm showed higher sensitivity (93%) than either Hcp1-ELISA or TTS1-PCR, for which sensitivity was 79% and 0%, respectively (see Tables S1 and S2 in the supplemental material).

**Prediction of melioidosis cases using AIM2 and FAM26F expression in nonbacteremic cases.** Next, we used our gene combination algorithm to predict which cases were likely to be melioidosis from the nonbacteremic enrollments. Of the 28 nonbac-
teremic cases, 15 were classified as likely to be melioidosis and 13 were unlikely to be melioidosis cases based on clinical grounds, patient outcomes, and antimicrobial treatment (Table 1). The mean age of likely cases was 53 ± 16 years, and for unlikely cases it was 45 ± 22 years. The ratio of males to females in both sample groups was 2:1. There was no statistically significant difference between the two groups for age or sex. The cases likely to be melioidosis showed a significantly longer duration of hospitalization than the cases unlikely to be melioidosis (P < 0.0001) (see Table S4 in the

![ROC Curve](image_url)

**FIG 3** Receiver operating curves (ROC) of AIM2 and FAM26F for septicemic melioidosis diagnosis. Expression levels of AIM2 and FAM26F in the training set of septicemic melioidosis cases and other sepsis cases were measured by real-time PCR, and ROC analysis was performed for selection of the cutoff values. The true-positive rate (sensitivity) is plotted against the false-positive rate (calculated as 1 – specificity) at various cutoff points. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a decision threshold.

<table>
<thead>
<tr>
<th>Area Under the Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
</tr>
<tr>
<td>AIM2</td>
</tr>
<tr>
<td>FAM26F</td>
</tr>
</tbody>
</table>

**TABLE 2** Cutoff determination based on receiver operating curve analysis of individual gene expressions from training set subjects

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cutoff value</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>NPV (%)</th>
<th>PPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIM2</td>
<td>0.033</td>
<td>100</td>
<td>62</td>
<td>100</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>0.061</td>
<td>83</td>
<td>85</td>
<td>85</td>
<td>83</td>
</tr>
<tr>
<td>FAM26F</td>
<td>0.015</td>
<td>83</td>
<td>92</td>
<td>86</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>0.020</td>
<td>75</td>
<td>100</td>
<td>81</td>
<td>100</td>
</tr>
</tbody>
</table>

*Bold indicates the cutoff values used to develop the cascading algorithm for melioidosis diagnosis.
*NPV, negative predictive value.
*PPV, positive predictive value.
Individual AIM2 and FAM26F expression levels were not significantly different between cases that were likely and unlikely to be melioidosis (see Fig. S3 in the supplemental material). The gene combination algorithm showed 40% sensitivity, 54% specificity, 44% NPV, and 50% PPV for prediction of cases likely to be melioidosis (Table 4).

**DISCUSSION**

Melioidosis presents in many forms, including acute, subacute, and chronic infections. Detection of *B. pseudomallei* by PCR assay has been applied to clinical samples, with some gene targets offering high specificity but variable sensitivity (32–35), as well as frequent false-negative results because of low pathogen levels in blood (36). Our study took a different approach to melioidosis diagnostics—that of looking at host gene expression changes rather than pathogen identification. We investigated host gene expression signatures using real-time PCR assays derived from microarray differential expression data that differentiated sepsis cases. Real-time PCR assays are more affordable as a diagnostic tool than microarray platforms for hospital laboratories at the national and regional levels.

The cost of testing for our melioidosis diagnostic gene combination algorithm was around $35 per test, much less expensive than the cost of a microarray assay but higher than that of latex agglutination ($1 to 3 per test). To be of value to the clinician, melioidosis test results should ideally be reported within 1 day of admission to help guide treatment. Treatment costs for severe melioidosis cases are much higher than the average costs for treating bacteremic melioidosis. One study estimated costs at $14,181 for treatment of fatal melioidosis cases against $1,515 for nonfatal cases (37). More than 80% of fatal cases result from the application of incorrect antibiotic regimens (38). If our method could reduce the number of improperly treated melioidosis cases, the savings in treatment costs would render the testing cost negligible. However, the financial cost

### TABLE 3

**Efficacy of the host gene expression cascade algorithm for differentiation of patients with bacterial culture-positive septicemic melioidosis from other bacterial sepsis cases**

<table>
<thead>
<tr>
<th>Set</th>
<th>No. of patients</th>
<th>AIM2(+) FAM266F(+)</th>
<th>AIM2(+) FAM266F(−)</th>
<th>AIM2(−)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>NPV (%)</th>
<th>PPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Training</td>
<td>B. pseudomallei cases (n = 12)</td>
<td>9</td>
<td>3</td>
<td>0</td>
<td>75</td>
<td>100</td>
<td>81</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Other cases (n = 13)</td>
<td>0</td>
<td>5</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Validation</td>
<td>B. pseudomallei cases (n = 21)</td>
<td>18</td>
<td>2</td>
<td>1</td>
<td>86</td>
<td>88</td>
<td>82</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Other cases (n = 16)</td>
<td>2</td>
<td>7</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All subjects</td>
<td>B. pseudomallei cases (n = 33)</td>
<td>27</td>
<td>5</td>
<td>1</td>
<td>82</td>
<td>93</td>
<td>82</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>Other cases (n = 29)</td>
<td>2</td>
<td>12</td>
<td>15</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

*AIM2(+) FAM266F(+), expression levels of AIM2 ≥ 0.033 and FAM266F ≥ 0.020 were interpreted as melioidosis positive.*
*AIM2(−), expression levels of AIM2 < 0.033 and FAM266F < 0.020 were interpreted as melioidosis negative.*
*NPV, negative predictive value.*
*PPV, positive predictive value.*

### TABLE 4

**Efficacy of the host gene expression cascade algorithm for differentiation between bacterial culture-negative patients likely and unlikely to be melioidosis**

<table>
<thead>
<tr>
<th>Bacterial culture-negative set</th>
<th>No. of patients</th>
<th>AIM2(+) FAM266F(+)</th>
<th>AIM2(+) FAM266F(−)</th>
<th>AIM2(−)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>NPV (%)</th>
<th>PPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Likely to be melioidosis (n = 15)</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>40</td>
<td>54</td>
<td>44</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Unlikely to be melioidosis (n = 13)</td>
<td>6</td>
<td>2</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*NPV, negative predictive value.*
*PPV, positive predictive value.*
for implementing our diagnostic gene combination algorithm requires more rigorous analysis.

The FAM26F single-gene assay differentiated between melioidosis and other bacterial septicemias but did not differentiate the septicemic melioidosis patients from healthy controls. The FAM26F and AIM2 cascade analysis showed comparative results to those of the FAM26F single-gene assay, but screening by AIM2 followed by FAM26F in the combination algorithm was able to exclude nonmelioidosis patients with 93% specificity. Additional data showed that melioidosis patients with severe sepsis had significantly downregulated expression of both AIM2 and FAM26F than other bacterial sepsis patients with or without septic shock (see Fig. S4 in the supplemental material). These results imply that the expression levels of both genes might relate to the severity of illness. Similar results from a study published in 2017 showed that the severity of acute pancreatitis correlated with expression of the AIM2 inflammasome (39). To verify how this finding relates to melioidosis, further investigation is needed.

The combination algorithm gave higher sensitivity than that of currently available diagnostic assays. Bacterial culture is the gold standard used in hospital laboratories throughout Thailand, and it is known that shorter times taken to achieve a positive culture correlate with improved clinical outcomes (40). Therefore, the 6-h test time offered by our real-time PCR assay would greatly benefit patient care. Of the 33 septicemic melioidosis patients in our study, 14 died, and 6 of these (43%) died within 2 days of admission. This is similar to a previous report that stated that 35% of melioidosis patients died within 2 days of admission (40). These mortality rates change substantially if the patient is given the melioidosis-appropriate drug regimen. For the 33 melioidosis patients in our study, 37% (10/27) of the patients treated with melioidosis-appropriate drug regimens within 48 h of admission died, versus a 67% (4/6) mortality rate for patients treated with inappropriate antibiotics. Our finding is consistent with those of two other studies that found that melioidosis mortality rates decreased from 74% to 37% (41) and 47% to 18.5% (42) when patients were treated with the appropriate drugs. These indicate that appropriate early treatment for melioidosis patients is crucial for their survival.

Attempts have been made to reduce the time to identification of bacterial culture isolates by, for example, the latex agglutination test. This method has been used to identify suspected B. pseudomallei colonies directly from culture plates, reducing the time to confirm the melioidosis diagnosis to 2 to 3 days after specimen collection. This latex method has high sensitivity and specificity; however, it has failed to have any clinical impact, and this method is only useful if the bacteria in hemoculture are enriched by incubation for 1 or 2 days (43).

Molecular methods amplifying B. pseudomallei genes in whole blood have been described with sensitivities ranging from 47% to 100% and specificities ranging between 67% to 100%, depending on the sequences targeted by the primers (20, 44–48). In one example, detection by PCR directed at a region of B. pseudomallei 16S rRNA in whole-blood extracts has shown high specificity (100%) but very low sensitivity (22.7%) (48), and, in our hands, real-time PCR failed to detect the B. pseudomallei TTS1 in blood samples. Detection of B. pseudomallei or other bacterial components in whole blood is difficult because the organism concentration is usually very low (21, 44), approximately 1.1 CFU/ml (21).

Currently, IHA is the method most commonly used for serodiagnosis of B. pseudomallei in areas where it is endemic, despite its low sensitivity and specificity (69.5% and 67.6%, respectively) and the lack of a standardized methodology (12). Other serological assays for detection of antibodies to B. pseudomallei have been described—for example, latex agglutination with polysaccharide-conjugated beads (12)—as well as ELISAs using different protein and polysaccharide antigens (14, 49). Serum IgG ELISAs show 72% to 83% sensitivity and >95% specificity, but it is impossible to separate recovering patients from those with active infections (14, 15). Multiplex protein arrays using both recombinant and purified B. pseudomallei proteins for serum IgG capture show >80% sensitivity and 97% specificity (22). However, they suffer from the same
limitation of all these serological assays in that antibodies can only be detected 7 to 14 days postinfection. While our algorithm showed higher sensitivity compared with that of the Hcp1-ELISA, the small number of samples tested means that further investigation is needed to establish the credibility of these comparisons.

Finally, rapid diagnostic lateral flow immunoassays (LFI) have been developed for use in laboratory settings where molecular and ELISA methods are generally not available. The Active Melioidosis Detect LFI (InBios International Inc., USA) detects soluble B. pseudomallei capsular polysaccharide and has high sensitivity and specificity (98.7% and 97.2%, respectively) when tested against B. pseudomallei isolates (17) but performs poorly when used with urine and blood samples (17–19). A recent study in Laos demonstrated that this LFI had high specificity when testing turbid blood culture bottles (100%), pus (100%), sputum (100%), sterile fluid (100%), and urine (90.7%). In addition, the LFI had 99% sensitivity on positive blood cultures; however, the sensitivity of LFI reduced dramatically when testing serum (13.9%) (50).

Both of our candidate genes (AIM2 and FAM26F) are involved in the innate immune response. The AIM2 inflammasome is activated by the presence of microbial DNA within the cytosol and induces the maturation of the proinflammatory cytokines interleukin 1β (IL-1β) and IL-18. It is essential for host cell defense. Previous studies confirm that B. pseudomallei, Salmonella spp., and Staphylococcus aureus activate AIM2 expression (24, 51, 52), and our data are consistent with this. FAM26F is a conserved, signal peptide-derived transmembrane protein that was recently described as a cation channel involved in the trafficking of molecules and ions within or between cells (53). It also appears to be a significant regulator of innate and/or adaptive immune responses (53). Increased FAM26F expression has been demonstrated in various cancers, as well as in viral, parasitic, and some bacterial infections, including those caused by B. pseudomallei (24). FAM26F has also been shown to be upregulated by lipopolysaccharide (LPS) through Toll-like receptor 4 (TLR4) stimulation (54). However, the differences in expression for B. pseudomallei compared to that for other Gram-negative infections needs elucidation. Therefore, there are two important caveats to the use of the AIM2 and FAM26F diagnostic algorithm for B. pseudomallei, namely, that (i) both of these genes also show upregulated expression in cancer (55–57), and (ii) a protein secreted in response to a common viral infection has been reported to inhibit AIM2 expression (58).

Our algorithm showed a 93% PPV in bacteremic cases, which is high for areas where melioidosis is endemic. Besides B. pseudomallei, the most common pathogens in northeast Thailand are the Gram-negative bacteria Escherichia coli and Klebsiella pneumoniae and the Gram-positive bacterium S. aureus (59), all of which our algorithm was able to successfully differentiate from B. pseudomallei. However, our method appeared to have difficulty differentiating melioidosis from other intracellular infections. Two of the 29 other bacterial infections in our study were falsely identified as B. pseudomallei positive by our algorithm, and both were Salmonella infections. The incidence of non-B. pseudomallei intracellular Gram-negative bacteria, especially Salmonella, is 5.1 cases/100,000 population (60), which equates to 4% of patients with a primary episode of community-acquired bacteremia in this area (61). Therefore, the impact of false-positive B. pseudomallei tests by our method is likely to be limited. Similarly, since viral infections can also activate the expression of both AIM2 and FAM26F, physicians will still need to refer to clinical signs and symptoms and laboratory results (complete blood cell count, dengue NS1Ag, and influenza rapid tests) to rule out viral infections. Further work is required to improve our algorithm so that it can distinguish other intracellular bacterial and viral infections from melioidosis. This could be facilitated in the future through a multiplex assay for rapid diagnosis.

In this study, patients with cancer or viral infection were excluded, and therefore further studies will be required to validate the AIM2 and FAM26F diagnostic signature as specific to melioidosis septicemia. Even with these apparent limitations, we believe that our diagnostic cascading algorithm using AIM2 and FAM26F expression would be of value to the clinician. Having a strong indicator that the infection was caused by B. pseudomallei within 24 h of hospital admission would allow for earlier initiation of
appropriate antibiotic treatment, which would be likely to reduce the mortality rate. This is particularly relevant for nonbacteremic melioidosis cases.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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We declare that we have no competing interests.

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