Longitudinal profiling of plasma cytokines in melioidosis and their association with mortality: a prospective cohort study

T Kaewarpai¹, P Ekchariyawat¹,², R Phunpang³, SW Wright⁴, A Dulsuk³, B Moonmueangsan¹,⁵, C Morakot⁶, E Thiansukhon⁶, NPJ Day³,⁷, G Lertmemongkolchai⁸, TE West⁹,¹⁰,¹#, N Chantratita¹,³,#,*

¹) Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand
²) Department of Microbiology, Faculty of Public health, Mahidol University, Bangkok, Thailand
³) Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand
⁴) Division of Pediatric Critical Care Medicine, Department of Pediatrics, University of Washington, Seattle, WA, USA.
⁵) Department of Medicine, Mukdahan Hospital, Mukdahan, Thailand
⁶) Department of Medicine, Udon Thani Hospital, Udon Thani, Thailand
⁷) Center of Tropical Medicine and Global Health, University of Oxford, Oxford, United Kingdom
⁸) Cellular and Molecular Immunology Unit, Centre for Research and Development of Medical Diagnostic Laboratories (CMDL), Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand
⁹) Division of Pulmonary Critical Care & Sleep Medicine, Harborview Medical Center, University of Washington, Seattle, WA, USA
¹⁰) International Respiratory and Severe Illness Center, University of Washington, Seattle, WA, USA

Abstract

Objectives: To characterize plasma cytokine responses in melioidosis and analyse their association with mortality.

Methods: A prospective longitudinal study was conducted in two hospitals in Northeast Thailand to enroll 161 melioidosis patients, 13 uninfected healthy controls, and 11 uninfected diabetic controls. Blood was obtained from all individuals at enrollment (day 0) and at days 5, 12, and 28.
from surviving melioidosis patients. IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A, IL-23, and TNF-α were assayed in plasma. The association of each cytokine and its dynamics with 28-day mortality was determined.

**Results:** Of melioidosis patients, 131/161 (81%) were bacteraemic, and 68/161 (42%) died. On enrollment median levels of IFN-γ, IL-6, IL-8, IL-10, IL-23, and TNF-α were higher in melioidosis patients compared with uninfected healthy controls and all but IFN-γ were positively associated with 28-day mortality. IL-8 provided the best discrimination of mortality (AUROCC 0.78, 95% CI 0.71–0.85). Over time, non-survivors had increasing IL-6, IL-8, and IL-17A levels, in contrast to survivors. In joint modeling, temporal trajectories of IFN-γ, IL-6, IL-8, IL-10, and TNF-α predicted survival.

**Conclusions:** In a severely ill cohort of melioidosis patients, specific pro- and anti-inflammatory and Th17 cytokines were associated with survival from melioidosis, at enrollment and over time. Persistent inflammation preceded death. These findings support further evaluation of these mediators as prognostic biomarkers and to guide targeted immunotherapeutic development for severe melioidosis.

**Keywords**
Cytokine; Biomarker; Outcome; Diabetes; Melioidosis

**Introduction**
Melioidosis, an infectious disease caused by the environmental saprophyte *Burkholderia pseudomallei*, is endemic across many areas of Southeast Asia, South Asia, Northern Australia and America (1). Patients may present with a wide range of clinical manifestations including pneumonia, bacteraemia, abscesses, and sepsis, frequently leading to multiorgan failure and eventually death. Risk factors for melioidosis include diabetes, high alcohol intake, renal disease, lung disease, liver disease and steroid therapy (2). Despite appropriate antibiotic treatment, the mortality rate from melioidosis exceeds 40% in certain Southeast Asian regions (1).

The immunological markers and pathways that identify patients at risk of death in melioidosis are not well understood. Several immune cells including macrophages, natural killer (NK) cells, NK T cells, and T cells respond to bacterial infection rapidly by generation of several potent cytokines (4). *B. pseudomallei* is a facultative intracellular bacterium, a characteristic that contributes to its pathogenesis and persistence in the host, and that may modulate the cytokine response to infection. In recent studies, we analyzed the *ex-vivo* human blood response of healthy individuals in Northeast Thailand and observed that *B. pseudomallei* and bacterial ligands lipopolysaccharide and flagellin induce significant inflammatory cytokine release (5–7). We also found that the blood cytokine responses to *B. pseudomallei* differ between individuals, and by age, sex and host genetics (5, 8–11). These data suggest that there is likely to be substantial variation in blood cytokine profiles in patients with melioidosis, and raise the possibility that these profiles may be useful in elucidating the pathogenesis of infection and serving as predictive biomarkers. The first objective of this study was to characterize the blood cytokine response in melioidosis over
the course of infection. The second objective was to test the hypothesis that specific plasma cytokine levels at enrollment, and changes in concentration over time, would distinguish patients who would not survive infection from those who would.

Methods

Study design and participants

A prospective longitudinal study of cytokine responses in 161 melioidosis patients was conducted at Udon Thani Hospital (UDH), Udon Thani, and Mukdahan Hospital (MDH), Mukdahan, Thailand between January 2015 and December 2018. This study was part of a larger multi-center study of melioidosis enrolling patients ≥15 years with culture-proven melioidosis. Potential study patients were identified by daily screening at each hospital diagnostic microbiology laboratory for any clinical samples that grew *B. pseudomallei* on culture. Inclusion criteria for melioidosis patients were: age ≥15 years, admitted to hospital and culture positive for *B. pseudomallei* within last 24 h and written informed consent/assent was obtained. Exclusion criteria were pregnancy, receiving palliative care, or incarceration. Blood samples were drawn at the time of enrollment (within 24 h of the culture result, defined as day 0) and days 5, 12 and 28 after enrollment. Plasma was separated at the study site and samples were frozen and shipped, with temperature monitoring, to a research laboratory for subsequent analysis. Clinical information was abstracted from the medical records. Mortality was ascertained in person or by telephone 28 days from the day of enrollment.

Uninfected control subjects, recruited from UDH to provide baseline immunologic data, included 13 healthy blood donors and 11 diabetic patients, male and female, age ≥18 years. Inclusion and exclusion criteria for these individuals are provided in the Supplementary Methods.

Ethics statement

Ethical approval was obtained from the ethical committee of Faculty of Tropical Medicine, Mahidol University (approval no. MUTM 2015-002-03), Udon Thani Hospital (approval no.6/2561) and Mukdahan Hospital (approval no. MEC 010/59). This study was conducted in accordance with the principles of the Declaration of Helsinki (2008) and the International Conference on Harmonization and Good Clinical Practice guidelines. The ethics committee approved the consent procedure. Written informed consent was obtained from all participants or their representatives.

Cytokine assays

Concentrations of IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A, IL-23, and TNF-α were determined in human plasma samples using bead-based multiplex assays of Luminex technology (MILLIPLEX® MAP kit: HSTCMAG-28K-12 plex, Millipore, MA, USA). The limits of detection for each cytokine are given in the Supplementary Methods. The number of subjects assayed for each cytokine at each time point is given in Supplemental Table 1.
**Statistical analyses**
Continuous variables were reported as mean and 95% confidence interval for normally distributed data and as median and interquartile range (IQR) for non-normally distributed data. Data were analyzed using the chi-squared test for categorical data, t-test for comparison of normally distributed continuous data, Mann-Whitney U test for comparison of non-normally distributed continuous unpaired data, and Wilcoxon signed-rank test for comparison of non-normally distributed continuous paired data. Discrimination of survivors and non-survivors was performed by quantifying the area under the receiver operating characteristic curve for each cytokine. Statistical significance was defined by a P-value < 0.05; correction for multiple testing on all 12 cytokines using the conservative Bonferroni method (0.05/12) yielded alpha = 0.004.

Serial cytokine concentrations (obtained at days 0, 5, and 12) were analyzed for association with 28-day survival using joint modelling as described in the Supplementary Methods. Analyses were performed using GraphPad Prism version 7 (San Diego, CA, USA) or using Stata SE v14.2 (StataCorp, College Station, TX, USA).

**Results**

**Characteristics of melioidosis patients**
One hundred and sixty one melioidosis patients were enrolled (Supplemental Figure 1). Thirteen healthy blood donors and 11 diabetic outpatients were enrolled as uninfected control groups. Demographic and clinical characteristics of the melioidosis patients are shown in Table 1. The median age was 54 years (IQR 45–64 years) and 114/161 (71%) were male. The most common underlying medical condition among melioidosis patients was diabetes (119/161, 74%). The majority of patients were bacteraemic (131/161, 81%).

**Clinical characteristics of melioidosis patients associated with 28-day mortality**
Sixty eight of the 161 melioidosis patients enrolled (42%) died within 28 days (Table 1). In comparison to survivors, patients who did not survive were more likely to have a history of stroke (0/93 (0%) vs 5/68 (7%), P=0.01); present with septic shock (17/93 (18%) vs 25/68 (37%), P<0.01); be admitted to the ICU (21/93 (23%) vs 46/68 (68%), P<0.001); receive mechanical ventilation (29/93 (31%) vs 58/68 (85%), P<0.001); receive circulatory support (15/93 (16%) vs 22/68 (32%), P=0.02); have bacteraemia (69/93 (74%) vs 62/68 (91%), P<0.01); or have pneumonia (31/93 (33%), vs 34/68 (50%), P=0.03).

**Plasma cytokine profiles in melioidosis patients on enrollment compared to control subjects**
Concentrations of 12 plasma cytokines were quantified in melioidosis patients at day 0 and compared to cytokines in uninfected healthy and uninfected diabetic controls (Figure 1 and Supplemental Table 1). For each cytokine the distribution in melioidosis patients encompassed at least one and up to three orders of magnitude. IFN-γ, IL-6, IL-8, IL-10, IL-23 and TNF-α were significantly higher in melioidosis patients compared to healthy controls (all P<0.004) and IFN-γ, IL-6, IL-8, and TNF-α were higher compared to diabetic controls (all P<0.004).
Plasma cytokine profiles of individual melioidosis patients over time

Trends in cytokine concentrations at days 0, 5, 12 and 28 in melioidosis patients were examined by calculating fold changes (Supplemental Table 2) and by plotting on a heatmap (Figure 2). Of the cytokines that were elevated in melioidosis patients on enrollment compared to uninfected controls, only IL-6 increased in all patients still alive on day 5 relative to day 0 (mean fold change 2.21, 95% CI 1.20–3.21). Visual inspection of the heatmap suggested that for many mediators the overall trend in concentration over time for survivors was downwards, although several individuals had progressive increase in concentrations of specific mediators at later stages. More non-survivors than survivors tended to have increased levels of IL-6, IL-8, IL-17A, and TNF-α at day 5 relative to day 0 although these did not reach statistical significance (Supplemental Table 2 and Supplemental Figure 2). In non-survivors still alive at day 12 the mean fold changes in IL-6 and IL-17A on day 12 compared to day 0 were significantly increased compared to survivors (P ≤0.004).

Plasma cytokine profiles of melioidosis patients on enrollment by clinical characteristics

Cytokine concentrations at day 0 were evaluated in patients with specific clinical characteristics. There were no differences in any cytokine based on diabetes status (Supplemental Figure 3). IL-10, IL-6, IL-8, and TNF-α were higher in patients with bacteraemia (all P<0.0001); IL-6, IL-8, and TNF-α were higher in patients presenting with hypotension or shock (all P<0.001); and IL-6 was higher in patients with pneumonia (P=0.0003).

Plasma cytokines of melioidosis patients on enrollment and with 28-day mortality

Cytokines in melioidosis patients at day 0 were compared between non-survivors and survivors to 28 days (Figure 3 and Supplemental Table 3). Non-survivors had significantly increased IL-6, IL-8, IL-10, and TNF-α compared with survivors (all P<0.001). In non-survivors, IL-23 was higher than in survivors but this did not quite meet statistical significance (P=0.005).

Receiver operating characteristics (ROC) plots of cytokines to discriminate death

To evaluate whether specific cytokines obtained at day 0 could discriminate between melioidosis patients who would survive or die, area under ROC curves (AUROCCs) were calculated (Figure 4). The highest AUROCCs were for IL-8 (0.78, 95% CI 0.71–0.85), IL-6 (0.75, 95% CI 0.67–0.82), and IL-10 (0.73, 95% CI 0.65–0.81).

Survival analysis of cytokine trajectory

To assess how cytokine trajectory over time may be related to death, cytokine concentrations obtained at days 0, 5, and 12 were examined. For example, the distinct profiles over time of IL-8 in individuals who survived to censoring and those who died are shown in Supplemental Figure 4. Survival analysis incorporating serial cytokine concentrations to represent cytokine trajectory was performed for each cytokine by shared parameter joint modelling for longitudinal and survival data using maximum likelihood. Co-variates included in each model were blood disorders, stroke, liver disease, age, and sex. Trajectories of IFN-γ, IL-10, IL-6, IL-8, and TNF-α were significant predictors of survival (P<0.004).
whereas IL-17A did not quite meet statistical significance (P=0.008) (Supplemental Table 4).

Discussion

The major findings of our study are as follows: There was considerable inter-individual variation in plasma cytokine responses of melioidosis patients. Enrollment levels of IFN-\(\gamma\), IL-6, IL-8, IL-10, IL-23 and TNF-\(\alpha\) were higher in melioidosis patients compared with uninfected controls. With the exception of IFN-\(\gamma\), these same cytokines were positively associated with 28-day mortality. In AUROCC analysis, IL-8 was the best cytokine for discrimination of survival status. Over time, non-survivors had increasing IL-6, IL-8, and IL-17A levels, in contrast to survivors. Temporal trajectories of IFN-\(\gamma\), IL-6, IL-8, IL-10, and TNF-\(\alpha\) were significant predictors of survival. Although diabetes is a major risk factor for melioidosis and is known to alter immune responses to infection, there were no differences in cytokine profiles based on diabetes status.

At enrollment and over time, and by a variety of analytic methods, our study clearly implicates IFN-\(\gamma\), IL-6, IL-8, IL-10, IL-17A, IL-23 and TNF-\(\alpha\) as markers of adverse outcomes from melioidosis. Many of these cytokines have been shown in other cohorts to be associated with severity and fatal outcomes in melioidosis (12, 13). IL-6, IL-10, and TNF-\(\alpha\) are significantly higher in pediatric patients with Gram-negative bacteraemia than those with Gram-positive bacteraemia (14), and many of these mediators have been evaluated as biomarkers of sepsis (15). IFN-\(\gamma\), a type II interferon, is produced by activated T cells and NK cells (16) and in murine models is essential for survival from melioidosis (17). IL-6, IL-8, IL-23 and TNF-\(\alpha\) are other pro-inflammatory cytokines produced by monocytes/macrophages, neutrophils and NK cells that participate in the host response to infection, including \textit{B. pseudomallei} infection (18, 19). In contrast, IL-10, an immunoregulatory cytokine with anti-inflammatory properties mainly produced by regulatory T cells and monocytes, may inhibit the IFN-\(\gamma\), IL-6, and TNF-\(\alpha\) responses to \textit{B. pseudomallei} as well as bacterial killing (20). A recent study also demonstrated the up-regulation of IL-17A, IL-17B and IL-23 expression in peripheral blood mononuclear cells of septic melioidosis patients, implicating a Th17 cytokine response (18). The involvement of these multiple mediators reflects the complexity of the pathogenesis of sepsis, a phenomenon that was exaggerated in this cohort of severely ill melioidosis patients.

The immune response in sepsis is highly variable and varies over time. The response may resolve or may be characterized by persistent inflammation or the development of immunosuppression (21). Although non-surviving patients had, on average, higher levels of several cytokines at day 0 than survivors, we observed significant inter-individual variability in cytokine responses, consistent with past observations of the whole blood innate immune response to \textit{B. pseudomallei} (5). Despite the initial degree of cytokine response, we found that non-surviving patients demonstrated significant subsequent increases in IL-6 and IL-17A compared to surviving patients, and we found an association of trends in IFN-\(\gamma\), IL-10, IL-6, IL-8, and TNF-\(\alpha\) with outcome using joint modeling. Thus, in melioidosis, death is typically preceded by a persistent or worsening inflammatory response, even after two weeks (or longer) of illness. These findings are broadly concordant with sepsis from
community-acquired pneumonia (22) but are important to define for melioidosis, given the facultatively intracellular nature of the pathogen, the diversity of disease, and the difficulty completely eradicating it from the host. Our data suggest that for melioidosis patients, approaches targeting persistent inflammation - assuming that this process is on the causal pathway toward death - may be beneficial. However, the persistent inflammation observed may alternatively reflect inadequate bacterial eradication/source control that necessitates additional drainage or antimicrobials. These possibilities should be evaluated in future studies.

It is well established that diabetes is a risk factor for melioidosis (23) and several studies demonstrate defects in immunity to B. pseudomallei in diabetics, such as impaired cellular responses (3) and neutrophils function (24). Despite this, diabetes is less robustly associated with outcome from melioidosis. Some studies indicate a protective effect of diabetes against death that may be attributable to the use of glyburide (25) but diabetes is not associated with hemostatic alterations in melioidosis (26). Any diabetes-related changes may be obscured by the significant immune abnormalities induced in septic melioidosis (26). This may be an explanation for not observing any significant differences in circulating cytokine concentrations between diabetic and non-diabetic patients.

There were some limitations to our study. Blood was obtained from patients only after the bacteria culture was confirmed, typically several days after admission. Thus, some severe patients may have died prior to enrollment. Despite this concern, the overall severity of illness was high; the large majority (81%) of patients was bacteraemic and the (42%) majority died within the 28 day. We did not include outpatients and probably do not capture the milder phenotypes of melioidosis. In comparison to other studies, the patients in our study were certainly more ill. For example, in another hospital in northeast Thailand, 43% of melioidosis cases in a 10-year prospective study died (27), and in Australia, 55% of melioidosis cases in a 10-year prospective study were bacteraemic and 14% died (28). In addition, there may be unmeasured confounders in our analyses that we did not consider.

In conclusion, our data identify specific pro- and anti-inflammatory and Th17 cytokines associated with survival from melioidosis, both at enrollment and over time. Persistent inflammation precedes death. These findings support further evaluation of these mediators as prognostic biomarkers and to guide targeted immunotherapeutics in melioidosis.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Plasma cytokine profiles of melioidosis patients at day 0, uninfected healthy controls, and uninfected diabetic controls.
Concentrations of IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17a, IL-23, and TNF-α, measured by bead-based multiplex assay, are shown on a log scale. The numbers of subjects for each cytokine are reported in Supplemental Table 1.
Figure 2. Heatmap of plasma cytokines in melioidosis patients at serial time points, uninfected healthy controls and uninfected diabetic controls. Concentrations of IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17a, IL-23, and TNF-α, measured at day 0, day 5, day 12 and day 28 in melioidosis patients and at a single time point in controls by bead-based multiplex assays. High concentrations of cytokines are shown in red whereas low concentrations are shown in green. The numbers of subjects for each cytokine at each time point are reported in Supplemental Table 1.
Figure 3. Plasma cytokine profiles of surviving and non-surviving melioidosis patients at day 0, by outcome at 28-days. Concentrations of IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17a, IL-23, and TNF-α, measured by bead-based multiplex assay, are shown on a log scale. The numbers of subjects for each cytokine and cytokine concentrations are reported in Supplemental Table 3.
Figure 4. Receiver operating characteristics (ROC) curves of plasma cytokines for discrimination of death at 28 days in melioidosis patients. The table shows areas under ROC curves and 95% CI. ($n_{\text{surviving melioidosis patients}}=93$), ($n_{\text{non-surviving melioidosis patients}}=68$).
### Table 1.

Clinical characteristics of melioidosis patients

| Characteristic                                      | All (n=161) | Survivors (n=93) | Non-survivors (n=68) | P-value |
|-----------------------------------------------------|-------------|------------------|----------------------|---------|
| **Demographics and pre-existing conditions**         |             |                  |                      |         |
| Age, median (IQR)                                   | 54 (45–64)  | 52 (44–61)       | 55.5 (47–65)         | 0.19    |
| Male gender (%)                                     | 114 (71)    | 63 (68)          | 51 (75)              | 0.32    |
| Charlson Comorbidity Index, median (IQR)            | 2 (1–3)     | 2 (1–3)          | 2.5 (1–4)            | 0.40    |
| Diabetes (%)                                        | 119 (74)    | 70 (75)          | 49 (72)              | 0.65    |
| Hypertension (%)                                    | 60 (37)     | 31 (33)          | 29 (43)              | 0.23    |
| Chronic kidney disease (%)                          | 31 (19)     | 18 (19)          | 13 (19)              | 0.97    |
| Blood disorders (%)                                 | 8 (5)       | 3 (3)            | 5 (7)                | 0.28    |
| Cardiovascular disease (%)                          | 7 (4)       | 4 (4)            | 3 (4)                | 1.0     |
| Stroke (%)                                          | 5 (3)       | 0                | 5 (7)                | 0.01    |
| Chronic lung disease (%)                            | 5 (3)       | 4 (4)            | 1 (1)                | 0.31    |
| Cancer (%)                                          | 5 (3)       | 2 (2)            | 3 (4)                | 0.65    |
| HIV infection (%)                                   | 4 (2)       | 2 (2)            | 2 (3)                | 1       |
| Chronic liver disease (%)                           | 3 (2)       | 1 (1)            | 2 (3)                | 0.57    |
| On immunosuppressive medications (%)                | 11 (7)      | 6 (6)            | 5 (7)                | 1       |
| **Clinical presentation**                           |             |                  |                      |         |
| Hypotension or shock (%)                            | 42 (26)     | 17 (18)          | 25 (37)              | <0.01   |
| **Clinical course**                                 |             |                  |                      |         |
| Received first line antimicrobial therapy (%)       | 156 (97)    | 90 (97)          | 66 (97)              | 1       |
| Underwent drainage procedure (%)                    | 57 (35)     | 36 (39)          | 21 (31)              | 0.31    |
| ICU admission (%)                                   | 67 (42)     | 21 (23)          | 46 (68)              | <0.001  |
| Required mechanical ventilation (%)                 | 87 (54)     | 29 (31)          | 58 (85)              | <0.001  |
| Required circulatory support (%)                    | 37 (23)     | 15 (16)          | 22 (32)              | 0.02    |
| **Site of infection**                               |             |                  |                      |         |
| Pneumonia (%)                                       | 65 (40)     | 31 (33)          | 34 (50)              | 0.03    |
| Bacteraemic pneumonia (%)                           | 54 (34)     | 23 (25)          | 31 (46)              | <0.01   |
| Urinary tract infection (%)                         | 23 (14)     | 13 (14)          | 10 (15)              | 0.87    |
| Arthritis or osteomyelitis (%)                      | 23 (14)     | 15 (16)          | 8 (12)               | 0.46    |
| Bacteraemia, any source (%)                         | 131 (81)    | 69 (74)          | 62 (91)              | <0.01   |
| **Distribution of melioidosis**                     |             |                  |                      |         |
| Localized (%)                                       | 22 (14)     | 19 (20)          | 3 (4)                | <0.01   |
| Multifocal (%)                                      | 8 (5)       | 5 (5)            | 3 (4)                | 1       |
| Non-disseminated (%)                                | 94 (58)     | 51 (55)          | 43 (62)              | 0.33    |
| Disseminated (%)                                    | 37 (23)     | 18 (19)          | 19 (28)              | 0.26    |

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a Continuous variables analyzed by Mann-Whitney U test;
b Categorical variables analyzed by chi-square test or Fisher’s exact test for values <10.

c Charlson Comorbidity Index includes age in calculation.

d Blood disorders include anemia, hemophilia, blood clots and blood cancer such as leukemia, lymphoma and myeloma.

e Hypotension or shock is defined as subjects with either a systolic blood pressure of less than 90 mmHg or requiring circulatory support at admission.

f Received first line antimicrobial therapy for melioidosis included individuals receiving ceftazidime, imipenem, or meropenem at the time of enrollment.

g Circulatory support defined as receipt of a continuous infusion of epinephrine, norepinephrine, dopamine or dobutamine at any time.

h Localized infection was a focus of infection without bacteraemia; multifocal infection was more than one focus of infection without bacteraemia; disseminated infection was more than one non-contiguous focus of infection with bacteraemia; non-disseminated infection was a single or no identifiable focus of infection with bacteraemia.