Retrospective Analysis of Fever and Sepsis Patients from Cambodia Reveals Serological Evidence of Melioidosis

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Abstract. *Burkholderia pseudomallei*, the etiologic agent of melioidosis, is predicted to be ubiquitous in tropical regions of the world with areas of highest endemicity throughout Southeast Asia (SEA). Nevertheless, the distribution of *B. pseudomallei* and the burden of melioidosis in many SEA countries remain unclear. In Cambodia, only two human endemic cases of melioidosis were reported through 2008 and since then only a few hundred cases have been described in the literature. This is in sharp contrast to the annual burden of thousands of cases in surrounding areas. To further investigate the prevalence of melioidosis in Cambodia, we used a recently developed O-polysaccharide-based rapid enzyme-linked immunosorbent assay to detect *B. pseudomallei*-specific antibodies in serum samples obtained from 1,316 febrile illness or sepsis patients from 10 different provinces. Based on a cutoff value derived through culture-confirmed melioidosis cases, the proportion of positive samples in our cohort was approximately 12%. Regression analysis indicated that the odds of obtaining a positive result were 2.2 times higher for males than females controlling for age and province (95% confidence interval: 1.6–3.2, $P < 0.001$). Consistent with this, 9.2% of females were positive versus 18.2% of males ($P < 0.001$). Notably, 22.5% of grain or rice farmers were positive versus 10.1% of subjects with occupations not involving regular contact with soil. Positive results varied significantly by province. Collectively, the results of this study suggest that the true burden of melioidosis in Cambodia is greater than has previously been reported.

INTRODUCTION

Burkholderia pseudomallei is an environmental saprophyte that causes melioidosis, a potentially fatal disease in humans and animals. This gram-negative bacterium is frequently isolated from soil and water in endemic regions, and human infections are typically acquired via percutaneous inoculation, inhalation, or ingestion.^{1–6} Individuals with repeated exposure to contaminated soil, water, or environmental aerosols are at particular risk of developing *B. pseudomallei* infection.⁷ The clinical presentation of melioidosis is variable and ranges from asymptomatic infections to acute pneumonia and severe sepsis.⁷ Treatment of melioidosis patients involves prolonged antibiotic regimens, and mortality rates associated with acute forms of the disease remain high.⁸

Although predicted to be globally ubiquitous in tropical regions of the world,⁹ *B. pseudomallei* is known to be highly endemic in many areas of Southeast Asia (SEA) where it causes significant morbidity and mortality.¹⁰ After the first identification of melioidosis in SEA in 1912,^{11–13} the first case was reported in Cambodia in 1930.¹⁴ Although cases of animal melioidosis and nonendemic human cases of melioidosis were reported,^{15–17} the next endemic case of human melioidosis in Cambodia was not reported until 2008.¹⁸ With most of the reported cases worldwide occurring in neighboring Thailand, the scarcity of melioidosis reports in Cambodia indicates that melioidosis is drastically underrepresented.^{1,8,9} This is due in part to insufficient laboratory capabilities throughout

the country. To date, only a few 100 cases of melioidosis in Cambodia have been described from a handful of studies since 2007, leaving the true distribution of *B. pseudomallei* and the burden of melioidosis unclear.^{19–26}

Serology studies have proven useful in providing epidemiological characterization of *B. pseudomallei* distribution.²⁷ The only recognized, clinically validated method of determining antibody titer is the indirect hemagglutination assay (IHA).^{28–30} Unfortunately, this method is burdensome and difficult to standardize, requires reagents that are often difficult to obtain in nonendemic areas, possesses poor sensitivity (56–70%), and is variably specific depending on the population being tested.^{31–33} As a result, some investigators have opted for more tractable assays such as the enzyme-linked immunosorbent assay (ELISA).^{34–36} Although evidence suggests that when properly validated, ELISAs can be a useful serological tool,³⁷ experts urge caution in the use of unvalidated assays for drawing conclusions on the seroprevalence of *B. pseudomallei* in an endemic population.^{37,38}

Recently, we developed a rapid O-polysaccharide (OPS)-based ELISA for the detection of antibodies to *B. pseudomallei* and validated it using serum from healthy and culture-confirmed melioidosis patients from Thailand.³⁹ The OPS component of lipopolysaccharide is an ideal capture antigen for use in serologic assays because it has been shown to be the dominant antigen against which human immune responses are directed after infection with *B. pseudomallei*.³⁹ We have previously used OPS as a target antigen in a rapid latex agglutination assay and demonstrated that it is a promising antigen for serodiagnosis of melioidosis, particularly in nonendemic areas.³³ When used as the capture antigen in our rapid ELISA format, OPS outperformed the gold

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FIGURE 1. Cambodia. An elevation map of Cambodia was generated using DIVA-GIS (www.diva-gis.org). Provinces where archived samples were originally obtained are shown in bold and surrounding areas are shown in grey and depicted as flat. Note: At the time samples were collected, Kampong Cham existed as a single, discrete province. However, Kampong Cham was recently divided along the Mekong River resulting in two provinces, Kampong Cham and Tboung Khmum. The map depicts provincial borders at the time the samples were collected.

standard IHA by a significant margin when using serum samples from both nonendemic and endemic regions. In the present study, we sought to improve our understanding of the risk and distribution of *B. pseudomallei* in Cambodia. To this end, we conducted a retrospective study in which we used our rapid OPS-ELISA to detect *B. pseudomallei*-specific antibodies in serum samples obtained from 1,316 patients with fever or sepsis of unknown origin from 10 different provinces of Cambodia.

MATERIALS AND METHODS

Samples. Serum samples were obtained from three studies conducted in 10 provinces throughout Cambodia (Figure 1). The study protocols were approved by the Naval Medical Research Center Institutional Review Board in compliance with all applicable federal regulations governing the protection of human subjects. Sera ($N = 986$) were derived from an acute febrile illness (AFI) study initiated in 2005 with collection sites established in suburban and rural areas throughout Cambodia. An additional 140 serum samples, including patients with culture-confirmed melioidosis, were derived from an observational study of sepsis in a single hospital in Takeo province, Cambodia, between 2014 and 2015.²⁶ The confirmed melioidosis patients were experiencing symptoms between 4 and 15 days (median: 5 days) before serum collection and have been thoroughly described.²⁶ Finally, 198 samples were also obtained from healthy volunteers with poultry contact who participated in a serosurvey of avian influenza A in

Kampong Cham province. In all cases, patients or –volunteers or their legal authorized representatives provided written informed consent and granted permission to store samples for future studies. A de-identified sample database was created for the present study with specimen ID, age, gender, occupation, and home demographic information.

ELISA. The rapid ELISA based on the detection of IgG antibodies against OPS antigen was performed as described previously.³⁹ The concentration of OPS antigen at 1 $\mu\text{g/mL}$ and serum dilution 1:2,000 were used. The absorbance value (optical density, OD) was determined at a wavelength 450 nm using a microtiter plate reader (BioTek, Winooski, VT).

Maps. Seropositivity map and population maps were generated using ArcGIS software version 10.3.1 (www.arcgis.com) whereas the elevation map was generated using DIVA-GIS (www.diva-gis.org).

Statistical analysis. A receiver operator characteristic (ROC) curve was constructed by GraphPad Prism 6 (GraphPad

TABLE 1
Sensitivity and specificity of OPS-ELISA

Cutoff (OD ₄₅₀)	Sensitivity (%)	Specificity (%)
	Melioidosis patients ($N = 7$)	Healthy donors ($N = 198$)
0.72	85.7	85.9
0.97	71.4	90.9
1.35	57.1	95.5

OD = optical density; OPS-ELISA = O-polysaccharide-based enzyme linked immunosorbent assay. Three cutoff values were calculated from patients who had melioidosis and from healthy donors.

FIGURE 2. Enzyme-linked immunosorbent assay results of four serum groups. The cutoff optical densities (ODs) used were set at 85% (OD 0.72), 90% (OD 0.97), and 95% (OD 1.35) specificity. The median line is presented within the boxes which encompass the 25th and 75th percentiles whereas the whiskers extend to the 10th and 90th percentiles.

Software, Inc., La Jolla, CA) as described previously.³⁹ The Mann–Whitney test was used to determine the difference between serum groups. The analyses of demographic characteristics were conducted using Stata version 14 (StataCorp, College Station, TX). Subjects seropositive for *B. pseudomallei* and seronegative subjects were compared using χ^2 or Student's *t* test. Odds ratios were estimated using logistic regression models. Information on occupation was missing for all subjects from Kampong Cham province. Tests of statistical significance were two-tailed, and in all cases, significance was defined as $P < 0.05$. Samples ($N = 19$) obtained in Phnom Penh (Figure 1, star) were included in data for Kandal province.

RESULTS AND DISCUSSION

In total, 1,316 samples from 10 provinces were used in this study. The gender was roughly evenly divided (52/48) between male and female. Thirty-seven percent were rice or grain farmers, 60% of whom (282) were male whereas 20% did not specify an occupation. The median age was 23 years (range 2–83). Included with these samples were controls collected from 198 healthy volunteers in Kampong Cham province (Figure 1). Thirty percent of these individuals were male, the median age was 41 (range 23–75), and they were enrolled between March 15 and November 1, 2011.

ROC analysis of OPS-ELISA. ROC curves were plotted by calculating the sensitivity and specificity of increasing numbers of the true-positive rate and false-positive rate ($1 - \text{specificity}$) from the results of OPS-ELISA using the culture-confirmed melioidosis group ($N = 7$, including one non-bacteremic case) and healthy Cambodian donors ($N = 198$). The area under the ROC curves for predicting past or present *B. pseudomallei* infection was used to determine our

cutoff value (Figure 3). Cutoff ODs used were set at 0.72, 0.97, and 1.35 to obtain 85%, 90%, and 95% specificity, respectively (Table 1).

The ability of these cutoffs to discriminate between the different serum groups (i.e., confirmed melioidosis, suspected sepsis, AFI, and healthy donors) was then evaluated at each of the potential cutoff values. The results depicted in Figure 2 demonstrate that a statistically significant difference could be

FIGURE 3. Area under the receiver operating characteristic curve of the O-polysaccharide-based enzyme-linked immunosorbent assay (OPS-ELISA) results of serum samples from the culture-confirmed melioidosis group and healthy Cambodian donors. The serum samples were diluted at 1:2,000 for OPS-ELISA.

FIGURE 4. Seropositivity by province. The overall and provincial incidences of seropositivity are presented using scatter plots. The cutoff value of OD_{450} of 0.905 is indicated with a dashed line. Samples that fall above that cutoff value were considered positive. The values for positive controls, negative controls, and blanks for each enzyme-linked immunosorbent assay plate are also presented. This figure appears in color at www.ajtmh.org.

observed when comparing each illness group to healthy donors. Furthermore, the results from the AFI and sepsis patients also demonstrated a statistically significant difference from the melioidosis group. Finally, the cutoff value with the highest sensitivity and specificity was determined to be 85.71% and

89.39%, respectively, and OD_{450} of 0.905 based on its discriminating power at 1:2,000 dilution (Figure 3).

Although these results suggest cutoff values of a highly sensitive and specific assay capable of identifying melioidosis patients in a cohort of unknown illnesses, the number of

TABLE 2
Burkholderia pseudomallei seropositivity in 10 Cambodian provinces

Province	Number	Farmer/other	Sex (M/F)	Median age (range)	Positive (%)	Odds ratio	P value	95% CI
Kampong Cham†	198	UNK	60/138	42 (23–75)	11	2.75	0.185	0.62–12.31
Kampong Speu	360	147/213	232/128	19 (2–66)	14	6.76	0.009	1.60–28.57
Kampot	20	10/10	16/4	53 (31–80)	25	3.76	0.143	0.64–22.15
Kandal	235	51/184	116/119	22 (2–78)	12	5.36	0.025	1.24–23.28
Kratie	65	29/36	20/45	25 (2–68)	8	3.55	0.141	0.66–19.29
Prey Veng	20	5/15	10/10	19 (5–60)	15	7.04	0.045	1.04–47.65
Ratanakiri	95	40/55	49/46	16 (2–61)	2	*	*	*
Stung Treng	73	31/42	35/38	22 (3–62)	15	7.17	0.013	1.51–33.99
Svay Rieng	67	17/50	36/31	25 (4–67)	27	13.94	0.001	3.05–63.66
Takeo	183	74/109	114/69	33 (2–83)	21	6.01	0.017	1.38–26.20
Total	1,316	402/712	698/635	23 (2–83)	12.3	N/A	N/A	N/A
Age	N/A	N/A	N/A	N/A	N/A	1.04	< 0.001	1.03–1.05
Gender	N/A	N/A	N/A	N/A	N/A	2.24	< 0.001	1.56–3.22

CI = confidence interval; N/A = not applicable; UNK = unknown. The details and results of the samples tested in each province are presented. In addition, the logistic regression analysis shows the odds of having antibodies against *Burkholderia pseudomallei* vary significantly by province, increase with age, and are more than two times higher for males compared with females.

* Ratanakiri is the comparison province.

† Samples collected in Kampong Cham were collected from healthy volunteers.

confirmed melioidosis patients used to generate these cutoffs was relatively low ($N = 7$). However, our previous work using Thai sera used a cutoff OD₄₅₀ of 0.87, which demonstrated a sensitivity and specificity of 71.6% and 96.7%, respectively.³⁹ Because our cutoff OD₄₅₀ of 0.905 is more stringent than the results generated in neighboring Thailand, we sought to estimate the prevalence of antibodies to *B. pseudomallei* as an indicator of potentially undiagnosed *B. pseudomallei* infections in Cambodia using this value.

Seropositivity in Cambodia. Overall, 163 of 1,316 (12%) samples tested fell above the cutoff value (OD₄₅₀ $\geq$ 0.905) indicating a high likelihood of *B. pseudomallei* infection (Figure 4). Regression analysis (Table 2) indicated the odds of *B. pseudomallei* seropositivity were 2.2 times higher for males than for females controlling for age and province (95% confidence interval [CI]: 1.6–3.2, $P < 0.001$). Consistent with that, 9.2% of females were seropositive versus 18.2% of males ($P < 0.001$). Notably, 22.5% of grain or rice farmers were seropositive versus 10.1% of subjects with other occupations. The median age was 21 for seronegative subjects and 33 for seropositive subjects ($P < 0.001$).

Burkholderia pseudomallei seropositivity varied significantly by province (Figures 4 and 5A), even when accounting for age and sex (Table 2). For example, the odds of being seropositive were nearly 14 times higher for subjects in Svay Rieng province compared with subjects from Ratanakiri (95% CI: 3.0–63.6, $P = 0.001$) with the lowest prevalence in Ratanakiri ($P < 0.001$). Seropositivity followed predictable elevation-dependent trends displaying an inverse relationship with elevation (spearman's $\rho = -0.67$, $P = 0.032$). For example, mountainous regions such as Ratanakiri (Figure 1) had the lowest frequency of positive samples (Figure 5A) whereas low-lying regions such as Svay Rieng and Takeo (Figure 1) had the highest rates of seropositivity (Figure 5A). Conversely, the frequency of positive samples had no correlation with population (spearman's $\rho = -0.03$, $P = 0.934$, compare Figure 5A and B) as areas such as the lightly populated Svay Rieng province presented the highest rates of seropositivity for example.

It is important to note that our samples are derived from a highly endemic region for melioidosis where prior exposure to *B. pseudomallei* in the population is likely quite high. Although culture remains the gold standard for diagnosis of melioidosis,

rapid serological assays represent an attractive complementary approach. Investigators have been working toward development of alternative diagnostic methods, and evidence suggests that when properly validated, ELISAs can be a useful tool for detecting exposure to *B. pseudomallei* and predicting active infections.³⁷ However, it is important that these assays be validated in a well-defined collection of samples before drawing conclusions on the seroprevalence of *B. pseudomallei* in an endemic population.³⁸ The antigens and methods used here have been systematically validated on clinical samples in direct comparison to the gold standard serological test IHA.^{33,39,40} We have previously shown that *B. pseudomallei* Type A OPS is a promising target antigen for serodiagnosis in different groups of melioidosis patients.³⁹ Our ELISAs have routinely demonstrated increased sensitivity and specificity and are proving to be simple serological screening tools for detection of *B. pseudomallei* infections.

Although this work represents the most comprehensive epidemiological survey of melioidosis in Cambodia to date, there is always room for improvement. Our study populations were derived from studies with vastly different inclusion criteria, each Province was not evenly represented and large portions of Cambodia remain unrepresented. To continue to improve our understanding of melioidosis in Cambodia, future epidemiological surveys should emphasize harmonized patient inclusion criteria and data collection in an all-inclusive nation-wide approach.

CONCLUSIONS

We analyzed 1,316 samples from 10 provinces and found serological evidence of *B. pseudomallei* infection in approximately 163 individuals. This work, coupled with recent prospective and retrospective studies,^{19–26} demonstrates that melioidosis represents a considerably higher burden in Cambodia than the present scientific literature would suggest. More studies are needed to raise awareness of melioidosis and continue to define the actual morbidity and mortality of this severe infectious disease.

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FIGURE 5. Maps. The provincial rates of seropositivity (A) and population (B) were mapped using ArcGIS software version 10.3.1 (www.arcgis.com) Note: Recently, Kampong Cham province was divided into two distinct provinces—Kampong Cham and Tboung Khmum—roughly along the Mekong River. However, at the time these samples were collected, Kampong Cham existed as a single, discrete province. Maps reflect provincial borders at the time of sample collection.

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