

## Antibodies in Melioidosis: The Role of the Indirect Hemagglutination Assay in Evaluating Patients and Exposed Populations

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**Abstract.** Melioidosis is a major neglected tropical disease with high mortality, caused by the Gram-negative bacterium *Burkholderia pseudomallei* (Bp). Microbiological culture remains the gold standard for diagnosis, but a simpler and more readily available test such as an antibody assay is highly desirable. In this study, we conducted a serological survey of blood donors ( $n = 1,060$ ) and adult melioidosis patients ( $n = 200$ ) in northeast Thailand to measure the antibody response to Bp using the indirect hemagglutination assay (IHA). We found that 38% of healthy adults (aged 17–59 years) have seropositivity (IHA titer  $\geq 1:80$ ). The seropositivity in healthy blood donors was associated with having a declared occupation of rice farmer and with residence in a nonurban area, but not with gender or age. In the melioidosis cohort, the seropositivity rate was higher in adult patients aged between 18 and 45 years (90%, 37/41) compared with those aged  $\geq 45$  years (68%, 108/159,  $P = 0.004$ ). The seropositivity rate was significantly higher in people with diabetes ( $P = 0.008$ ). Seropositivity was associated with decreased mortality on univariable analysis ( $P = 0.005$ ), but not on multivariable analysis when adjusted for age, diabetes status, preexisting renal disease, and neutrophil count. This study confirms the presence of high background antibodies in an endemic region and demonstrates the limitations of using IHA during acute melioidosis in this population.

### INTRODUCTION

Melioidosis, a major cause of fatal community-acquired sepsis, is an increasing global public health concern, with an estimated 89,000 deaths per annum across tropical regions throughout the world.<sup>1,2</sup> This disease is caused by *Burkholderia pseudomallei* (Bp), a Gram-negative soil-dwelling bacillus naturally found in the soil of rice paddies and stagnant water. People acquire infection through direct skin contact, inhalation, or ingestion of contaminated water, and most of the clinical cases have at least one risk factor for melioidosis, such as diabetes, preexisting renal disease, alcohol excess, or old age.<sup>3,4</sup>

Repeated natural exposure to Bp gives rise to detectable levels of specific antibodies in blood, although these antibodies may not be protective.<sup>5–8</sup> The indirect hemagglutination assay (IHA) remains a widely used serological test for clinical epidemiology and case detection as it is cheap and relatively easy to perform. However, a high seropositive rate in healthy individuals living in highly endemic areas has been reported,<sup>7–9</sup> and it has been hypothesized that such seropositivity may be due to cross-reactivity of IHA responses to avirulent soil *Burkholderia* species such as *Burkholderia thailandensis* (Bt). Studies have demonstrated that the IHA alone is insufficient for diagnosis and defining exposure to Bp because of its low specificity and sensitivity.<sup>10,11</sup> Nevertheless, IHA is still used as a marker of exposure to Bp, so here we provide new data for the interpretation of IHA.

It has been assumed that individuals who are regularly exposed to contaminated soil are more likely to have increased anti-Bp antibody levels, but few formal reports have been

published so far. This study therefore aimed to evaluate the relationship of IHA seropositivity and the demographic profiles of healthy blood donors living in Ubon Ratchathani, an endemic province in northeast Thailand. The demographic profiles included occupation as rice farmer and residence in nonurban areas.

There is a lack of data on the relationship between seropositivity and diabetes status, a major preexisting condition, in adult Asian patients with melioidosis. In this study, we then examined the association between IHA seropositivity and survival, diabetes status, and age in a unique longitudinal cohort of adult patients with culture-confirmed melioidosis. We also explored the 52-week dynamic of serological profiles in patients who survived the disease.

### MATERIALS AND METHODS

**Study populations.** Two cohorts of serum samples were used in the study. The endemic population cohort included serum samples obtained from 1,060 blood donors visiting the blood bank mobile units of Sunpasitthiprasong Hospital setup across Ubon Ratchathani Province, northeast Thailand, within 2006. The melioidosis patient cohort included serum samples collected from 200 adult in-patients with culture-confirmed melioidosis (age  $\geq 19$  years) at Sunpasitthiprasong Hospital between October 2012 and September 2014.<sup>12</sup> The patients were enrolled into the study following positive culture of Bp in any clinical specimen, which was a median of 5 days (interquartile range [IQR] 3–6, range 2–13) after admission. One quarter (51/200) of melioidosis patients died within 28 days after admission. Two patients were lost to follow-up, and their mortality status is unknown; hence, they were excluded from all mortality analyses. Among 149 surviving patients in the cohort, 103 (69%) participants underwent complete follow-up with sample collection at 12 and 52 weeks

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after enrollment. Each participant's residence was designated "urban" if located within a metropolitan district or main city of the province, or "nonurban" if located outside these areas. Occupational information was available for 822/1,060 (77.5%) of the healthy cohort. Three hundred sixty people reported their occupation as rice farmer, whereas other occupations reported included government officer ( $n = 130$ ), laborer ( $n = 128$ ), student ( $n = 67$ ), housewife ( $n = 39$ ), businessperson ( $n = 38$ ), monk ( $n = 17$ ), fisherman ( $n = 1$ ), or other employee ( $n = 42$ ).

Ethical approval for the study was obtained from three institutional review boards at the Faculty of Tropical Medicine, Mahidol University (Submission number TMEC 12-014), at Sunpasitthiprasong Hospital, Ubon Ratchathani (reference 018/2555), and The Oxford Tropical Research Ethics Committee (reference 64-11).

**Indirect hemagglutination assay.** Titers of antibodies against *Bp* were assessed by the IHA protocol of Mahidol-Oxford Tropical Medicine Research Unit,<sup>13</sup> as modified from a protocol previously described.<sup>7,14</sup> Briefly, *Bp* clinical isolates 199a and 207a originating from patients with melioidosis in northeast Thailand were cultured separately before being heat-killed at 121°C for 15 minutes. Two clinical strains rather than one that were used as different strains of *Bp* show a wide degree of genetic diversity, and antigenic variation is likely.<sup>15,16</sup> Concentration of each antigen preparation was standardized with reference pooled sera before use in the assay to prevent batch-to-batch variation. Optimal concentration of each antigen was then pooled before sensitizing with sheep red blood cells for 1 hour. Sensitized red blood cells were then mixed with 2-fold dilutions starting from a dilution of 1:10 of heat-inactivated serum. The mixture was incubated at room temperature for 2 hours before overnight incubation at 4°C. Plates can be reliably read at either time point. The highest antibody dilution demonstrating complete or partial agglutination was recorded as the IHA titer (e.g., 1:80 or 1:160).

**Statistical analysis.** Non-normally distributed data were reported as median and IQR. The significance of differences between two groups was analyzed by Mann-Whitney *U*-test in GraphPad Prism 7 for Windows (GraphPad Software, San Diego, CA). We estimated the odds ratio (OR) and multivariable logistic regression adjusting for age, gender, diabetes, preexisting renal disease, and neutrophil count<sup>12</sup> using Stata 14.0 statistical software StataCorp LLC, College Station, TX).

Goodness of fit and the area under the receiver operating characteristic curve (AUROC) were performed for each multivariable logistic regression model. A two-tailed *P* value < 0.05 was considered significant.

## RESULTS

**Serological surveys of anti-*Bp* antibodies in the endemic population cohort.** We measured levels of anti-*Bp* antibodies by IHA in the serum of 1,060 healthy volunteer blood donors with no known history of melioidosis. Subjects were aged from 17 to 59 years (median 37, IQR 30–42), and 44% were male. Seventy percent of the healthy cohort had a detectable IHA titer ( $\geq 1:10$ ). An IHA titer of greater than or equal to 1:80 was considered a *Bp*-seropositive result and was found in 403/1,060 (38%) of individuals. As an IHA titer of 1:160 has also been used as a cutoff titer for the diagnosis of clinical melioidosis in Thailand,<sup>7</sup> we also calculated the percentage of individuals who have an IHA titer of 1:160 or greater. We found that the proportion of *Bp*-seropositive population with the  $\geq 1:160$  cutoff was 298/1,060 (28%).

The *Bp*-seropositivity rate was not statistically different between adults aged < 45 years and adults aged  $\geq 45$  years<sup>1,11</sup> ( $P = 0.81$ , Table 1). We found a similar pattern when we used a cutoff threshold titer of 160 (data not shown). However, we found that participants who lived in nonurban districts were around 3-fold more likely to be *Bp* seropositive (IHA titer  $\geq 1:80$ ) than those who lived in urban districts (OR 2.8, 95% confidence interval [CI]: 2.2–3.7; Table 1). People who worked as a rice farmer were 4.6 times more likely to be seropositive than those who had other occupations such as businessperson, fisherman, laborer, housewife, student, and monk (OR 4.6, 95% CI: 3.4–6.2; Table 1).

**Correlation between seropositivity and patient characteristics in the melioidosis patient cohort.** The proportion of melioidosis patients, age ranging from 19 to 88 years (median 56, IQR 46–63), with any detectable IHA titer ( $\geq 1:10$ ), was 80% (159/200) during the acute illness (week 0, a median of 5 days post admission), and 72.5% (145/200) had *Bp* seropositivity as defined by an IHA titer  $\geq 1:80$ . The percentage of seropositive patients was 60% (119/200) when using a titer of 1:160 as a cutoff titer.

TABLE 1  
Seroprevalence of 1,060 healthy blood donors enrolled into the endemic population cohort in 2006

Characteristics	Seronegative (IHA < 1:80) <i>n</i> (%)	Seropositive (IHA $\geq 1:80$ ) <i>n</i> (%)	Crude OR (95% CI)
All participants	<i>N</i> = 657	<i>N</i> = 403	
Gender*			
Female	240/430 (56%)	169/297 (57%)	1.0
Male	190/430 (44%)	128/297 (43%)	1.0 (0.7–1.3)
Age (years)†			
< 45	351/431 (81%)	244/297 (82%)	1.0
$\geq 45$	80/431 (19%)	53/297 (18%)	1.0 (0.6–1.4)
Residence			
Urban	316/657 (48%)	99/403 (25%)	1.0
Nonurban	341/657 (52%)	304/403 (75%)	2.8 (2.2–3.7)‡
Occupation§			
Others	363/523 (69%)	99/299 (33%)	1.0
Rice farmer	160/523 (31%)	200/299 (67%)	4.6 (3.4–6.2)‡

CI = confidence interval; IHA = indirect hemagglutination assay; OR = odds ratio.

\* Gender of 333/1,060 subjects are unknown.

† Age of 332/1,060 subjects are unknown.

‡  $P < 0.05$ .

§ Occupation of 238/1,060 are unknown.

In univariable analysis, we did not find a relationship between *Bp* seropositivity and gender, residence, bacteremia, or preexisting renal disease (Table 2). We found that adult patients aged  $\geq 45$  years were less likely to be seropositive (68%; 108/159) compared with those aged less than 45 years (90%; 37/41, crude OR 0.2, 95% CI: 0.08–0.7; Table 2). The association remained significant when we used a cutoff IHA titer of 1:160 (crude OR 0.3, 95% CI: 0.2–0.8).

Diabetes mellitus (67%; 134/200) was the major underlying condition associated with melioidosis in this cohort, comparable with previous studies.<sup>3,4</sup> We found that the melioidosis patients with diabetes were 2.6 times more likely to have an IHA titer of 1:80 or greater (crude OR 2.6, 95% CI: 1.4–5.0; Table 2), and the odds were increased to 3.9 times at a titer of 1:160 as a cutoff titer (crude OR 3.9, 95% CI: 2.1–7.2). We also found a weak relationship between IHA titer and glycated haemoglobin level, which identifies average plasma glucose concentration (Spearman rho 0.24, 95% CI: 0.10–0.37,  $P < 0.001$ ). However, for people with known diabetes, no relationship between IHA titer and medication used to control diabetes before admission (metformin, sulphonyl urea, and insulin) was observed in our cohort. We also found a significant relationship between seropositivity and preexisting renal disease (crude OR 0.4, 95% CI: 0.2–0.9; Table 2).

The neutrophil count on admission in patients who were seropositive (median 8,862 cells/ $\mu$ L, IQR 6,453–11,760) was significantly lower than that in patients who were seronegative (median 11,798 cells/ $\mu$ L, IQR 6,925–14,851,  $P = 0.03$ ). Patients having a circulating neutrophil count of  $\geq 12,000$  neutrophils/ $\mu$ L were less likely to be IHA seropositive compared with those having a normal neutrophil range of  $> 4,000$ – $8,000$  cells/ $\mu$ L (crude OR 0.3, 95% CI: 0.1–0.7; Table 2). We found the same association between seropositivity and neutrophil count when we used a cutoff of 1:160 (data not shown).

Indirect hemagglutination assay seropositivity remained significantly associated with age  $< 45$  years (adjusted OR 0.2,

95% CI: 0.1–0.8; Table 2), diabetes status (adjusted OR 2.6, 95% CI: 1.3–5.4), and preexisting renal disease (adjusted OR 0.4, 95% CI: 0.2–1.0) in the multivariable logistic regression model. Patients with a circulating neutrophil count of  $\geq 12,000$  cells/ $\mu$ L remained less likely to be seropositive when compared with those having a neutrophil count in the normal range (adjusted OR 0.2, 95% CI: 0.1–0.6; Table 2).

**Increasing IHA titer correlated with survival in the melioidosis patient cohort.** The IHA titer of melioidosis patients during the acute phase was significantly higher than that of healthy control subjects living in the same province ( $P = 0.009$ , Figure 1). The IHA titer of melioidosis patients who survived was significantly higher than that of patients who died ( $P = 0.004$ , Figure 1). Univariable analysis shows that patients with seropositivity were less likely to die than those with seronegativity (crude OR 0.4, 95% CI: 0.2–0.8; Table 3).

As shown in our previous study,<sup>12</sup> we reported the relationship between mortality and preexisting renal disease (crude OR 2.6, 95% CI: 1.2–5.7) in the univariable model. We also reported the “J-shaped curve” effect in the relationship between high mortality rate and low (crude OR 5.8, 95% CI: 1.2–27.0 for  $\leq 4,000$  neutrophils/ $\mu$ L) and high neutrophil counts (crude OR 8.7, 95% CI: 2.4–30.9 for  $> 8,000$ – $12,000$  neutrophils/ $\mu$ L, and crude OR 10.1, 95% CI: 2.8–36.4 for  $\geq 12,000$  neutrophils/ $\mu$ L) compared with the normal neutrophil range ( $> 4,000$ – $8,000$  neutrophils/ $\mu$ L).

The association between mortality and IHA seropositivity was of borderline significance in the multivariable logistic regression model (adjusted OR 0.5, 95% CI: 0.2–1.0; Table 3). In the multivariable analysis, the mortality remained significantly associated with preexisting renal disease (adjusted OR 3.1, 95% CI: 1.3–7.4) and neutrophil counts outside the normal range (adjusted OR 6.1, 95% CI: 1.2–30.3 for  $\leq 4,000$  neutrophils/ $\mu$ L; adjusted OR 10.9, 95% CI: 2.9–41.4 for  $> 8,000$ – $12,000$  neutrophils/ $\mu$ L; and adjusted OR 11.0, 95%

TABLE 2

Correlation between IHA seropositivity and demographic and clinical characteristics of 200 patients enrolled into the melioidosis patient cohort

Characteristics	Seronegative (IHA $< 1:80$ ) <i>n</i> (%)	Seropositive (IHA $\geq 1:80$ ) <i>n</i> (%)	Crude OR (95% CI)	Adjusted OR (95% CI)	<i>P</i> -value
All patients	<i>N</i> = 55	<i>N</i> = 145			
Gender					
Female	18/55 (33%)	49/145 (34%)	1.0	1.0	
Male	37/55 (67%)	96/145 (66%)	0.9 (0.5–1.8)	1.2 (0.6–2.6)	0.62
Age (years)					
$< 45$	4/55 (7%)	37/145 (26%)	1.0	1.0	
$\geq 45$	51/55 (93%)	108/145 (74%)	0.2 (0.08–0.7)*	0.2 (0.1–0.8)	0.02
Residence					
Urban	8/55 (15%)	16/145 (11%)	1.0	1.0	
Nonurban	47/55 (85%)	129/145 (89%)	1.4 (0.6–3.4)	1.2 (0.4–3.4)	0.69
Diabetes					
No diabetes	27/55 (49%)	39/145 (27%)	1.0	1.0	
Diabetes	28/55 (51%)	106/145 (73%)	2.6 (1.4–5.0)*	2.6 (1.3–5.4)	0.008
Preexisting renal disease					
Absent	40/55 (73%)	125/145 (86%)	1.0	1.0	
Present	15/55 (27%)	20/145 (14%)	0.4 (0.2–0.9)*	0.4 (0.2–1.0)	0.047
Bacteremia					
No bacteremia	24/55 (44%)	71/145 (49%)	1.0	1.0	
Bacteremia	31/55 (56%)	74/145 (51%)	0.8 (0.4–1.5)	1.1 (0.5–2.2)	0.87
Neutrophil count/ $\mu$ L <sup>12</sup>					0.007
$> 4,000$ – $8,000$ †	10/55 (18%)	45/145 (31%)	1.0	1.0	
$\leq 4,000$	7/55 (13%)	13/145 (9%)	0.4 (0.1–1.3)	0.6 (0.2–1.9)	
$> 8,000$ – $12,000$	13/55 (24%)	54/145 (37%)	0.9 (0.4–2.3)	0.9 (0.3–2.3)	
$\geq 12,000$	25/55 (45%)	33/145 (23%)	0.3 (0.1–0.7)*	0.2 (0.1–0.6)	

CI = confidence interval; IHA = indirect hemagglutination assay; OR = odds ratio.

\*  $P < 0.05$ .

† Normal neutrophil range.

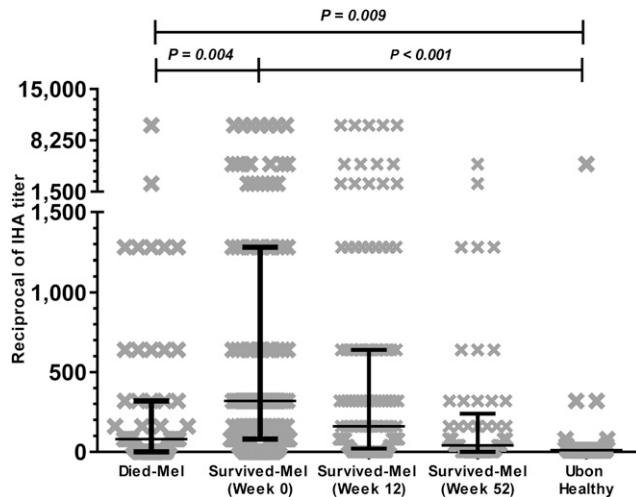


FIGURE 1. Anti-*Burkholderia pseudomallei* antibody levels in serum of adult in-patients with melioidosis at Sunpasithiprasong Hospital, Ubon Ratchathani, during 2012–2014 by using indirect hemagglutination assay (IHA). Survived-Mel and died-Mel refer to patients who survived and those who did not survive melioidosis, respectively. *P*-value by Mann-Whitney *U*-test.

CI: 2.9–42.5 for  $\geq 12,000$  neutrophils/ $\mu$ L). The AUROC of the mortality prediction was 0.7 (95% CI: 0.7–0.8) for this multivariable model.

**Dynamics of antibody responses to *Bp* in survivors overtime.** During acute melioidosis (week 0), 145/200 (72.5%) patients with culture-confirmed disease had an IHA  $\geq 1:80$ , of whom 114/145 (79%) went on to survive the infection, 29/145 (20%) were non-survivors, and 2/145 (1%) were lost to follow-up. Fifty-two weeks later, 65/103 (63%) of survivors for whom we had an IHA for all three time points (weeks 0, 12, and 52) remained IHA positive, with the median IHA titer decreasing over time (Figure 1, Table 4). The dynamics of the serological changes over 52 weeks for 103 survived subjects is shown in Table 4. Of the 103 survivors, 50 (49%) patients were persistently seropositive, whereas 13 (13%) patients remained seronegative (titers below 1:80) throughout the study.

## DISCUSSION

Despite limitations in sensitivity and specificity, the IHA remains a commonly used serological test for epidemiological

studies to assess exposure to *Bp*.<sup>6–8,14,17</sup> A previous study demonstrated that the IHA titers of all healthy donors living in non-endemic areas were less than 1:80; therefore, we used 1:80 as cutoff titer in this study.<sup>18</sup> The 38% seropositivity rate in our healthy endemic population cohort living in Ubon Ratchathani, the melioidosis-endemic region in northeast Thailand, was far greater than rates reported from northern Australia (6%), another hyperendemic region, even although they used a lower cutoff point at 1:40.<sup>19</sup> A previous study showed that 75–80% of children ( $\leq 4$  years old) living in Ubon Ratchathani have a detectable IHA titer of  $\geq 1:10$ ,<sup>5,7</sup> comparable with those in healthy adults (70%) in this present study. People living in this region may be exposed to *Bp* or other closely related nonpathogenic *Burkholderia* species that co-exist with *Bp* such as *Bt*<sup>20–22</sup> and develop a detectable antibody level from a young age, with antibody responses persisting into adulthood because of repeated natural exposure to environmental *Burkholderia* species. Nevertheless, the existence of cross-reactive antibody responses has not been clearly established, since previous studies have not been able to demonstrate cross-reactive IHA titers between *Bp* and *Bt* in healthy controls.<sup>23,24</sup> We are presently exploring the contribution of cross-reactivity to antibody responses in our laboratory.

We report data to formally support the widely held assumption that IHA seropositivity in healthy adults is significantly associated with occupation as a rice farmer and with residence in nonurban areas. Our results provide quantitative data to support the hypothesis that as *Bp* naturally inhabits soil and rice paddies, people living in rural areas and working in rice fields are more likely to be repeatedly exposed to this microorganism.

A limitation to this study is that information on place of residence of individuals was obtained from their official registration, which in some cases may not be up to date and may show their hometown, not their current residence. The relationship between high IHA titers and living location is likely in many cases to reflect exposure to *Bp* when they were at their hometown rather than current exposure.

In our melioidosis cohort, only 72.5% of patients with acute culture-confirmed melioidosis were seropositive. Thirteen percent of survivors had a negative IHA result throughout the study despite their clinical specimens testing positive for *Bp* culture, indicating the limits to the sensitivity of IHA. Our dataset also demonstrated that the dynamics of the seropositivity rates vary between individuals, with some culture-confirmed cases of melioidosis seroreverting to negative after

TABLE 3  
Multivariable-adjusted logistic regression for mortality of 198 adult patients with acute melioidosis

Variables	Patients who survived, n (%)	Patients who died, n (%)	Crude OR (95% CI)	Adjusted OR (95% CI)	<i>P</i> -value
All patients	N = 147	N = 51			
Seropositive (indirect hemagglutination assay $\geq 1:80$ )	114/147 (78%)	29/51 (57%)	0.4 (0.2–0.8)*	0.5 (0.2–1.0)	0.05
Age $\geq 45$ years	115/147 (78%)	42/51 (82%)	1.3 (0.6–2.9)	0.7 (0.3–1.9)	0.56
Diabetes	101/147 (69%)	32/51 (63%)	0.8 (0.4–1.5)	0.8 (0.4–1.7)	0.61
Preexisting renal disease	20/147 (14%)	15/51 (29%)	2.6 (1.2–5.7)*	3.1 (1.3–7.4)	0.01
Neutrophil count/ $\mu$ L <sup>12</sup>					0.001
> 4,000–8,000†	52/147 (35%)	3/51 (6%)	1.0	1.0	
$\leq 4,000$	15/147 (10%)	5/51 (10%)	5.8 (1.2–27.0)*	6.1 (1.2–30.3)	
> 8,000–12,000	44/147 (30%)	22/51 (43%)	8.7 (2.4–30.9)*	10.9 (2.9–41.4)	
$\geq 12,000$	36/147 (24%)	21/51 (41%)	10.1 (2.8–36.4)*	11.0 (2.9–42.5)	

CI = confidence interval; OR = odds ratio.

\* *P* < 0.05.

† Normal neutrophil range.

TABLE 4

Seropositivity status (IHA titer  $\geq 1:40$ ) of survivors ( $n = 103$ ) at three time points after admission with culture-confirmed melioidosis, where week 0 is a median 5 days (range 2–13) after admission to hospital with clinical melioidosis

Week 0	Week 12	Week 52	Interpretation	N (%) of 103 obtained IHA
+	+	+	Persistently seropositive	50 (48.5%)
+	+	–	Late seroreversion	14 (13.6%)
+	–	–	Early seroreversion	8 (7.8%)
+	–	+	Transient seroreversion	8 (7.8%)
–	+	–	Transient seropositivity	3 (2.9%)
–	–	+	Late seroconversion	2 (1.9%)
–	+	+	Seroconversion	5 (4.9%)
–	–	–	Persistently seronegative	13 (12.6%)

IHA = indirect hemagglutination assay.

12 (8%) or 52 weeks (14%) after admission, or showing transient seroreversion (8%). Some cases with IHA negativity at presentation were subsequently seropositive after 12 (5%) or 52 weeks (2%), or showed transient seroconversion (3%). Such variation in the pattern of IHA responses was also reported in the Australian studies.<sup>6,11</sup> We found no evidence to support the use of the IHA test in monitoring response to therapy (e.g., monitoring of syphilis serology to determine treatment effectiveness) because the IHA titer in the melioidosis cohort did not clearly relate to disease burden, and 38% of healthy adults in the region with no evidence of clinical melioidosis had positive IHA titers above 1:80. This study was unable to evaluate whether positive titers represent latent infection or immunity to past infection. In a non-endemic region, a positive IHA titer could prompt the physician to consider the risk of latent disease before administering immunosuppressive therapy, but the result may represent past, cleared infection or cross-reactivity to avirulent species and is therefore not reliable. A negative titer would not rule out the potential for latent disease because this study demonstrates that some people never seroconvert in spite of culture-confirmed melioidosis.

We did not find the negative correlation between IHA titer and bacteremia ( $P = 0.5$ ) reported in the Australian study.<sup>6,11</sup> This may reflect the IHA assay in our study being performed on serum taken a median of 5 days after admission (once culture-confirmation of melioidosis had been made), which gave more time for the development of an antibody response in the days since admission when the blood culture sample was typically obtained. Other factors including bacterial strain differences may be relevant.

Eighty-one percent of our melioidosis cohort had at least one risk factor including diabetes, preexisting renal disease, alcohol abuse, or age older than 65 years.<sup>25</sup> Indirect hemagglutination assay seropositivity rates were lower in patients with renal disease, and in people aged 45 years or older in a multivariable analysis adjusting for age, gender, diabetes, and preexisting renal disease (Table 2). Patients with these underlying conditions are considered to be immunocompromised,<sup>26,27</sup> and this is associated with lower specific antibody titers, and hence leading to negative results in serodiagnostic assays despite culture confirmation of *Bp* infection. The IHA test is therefore unsuitable for the diagnosis of melioidosis in patients with risk factors associated with immune suppression. It is possible that antibodies specific to *Bp* are induced after infection in all people, but cannot bind with antigens used in IHA test.

We found a significant relationship between IHA seropositivity and diabetes status using a multivariable regression

model including age, gender, and preexisting renal disease. A previous study in northern Australia<sup>11</sup> reported a similar relationship, which was not seen for an enzyme-linked immunosorbent assay (ELISA)-based assay. Authors of the Australian study have postulated that the immunosuppression that occurs in diabetes results in more indolent presentations of melioidosis caused by less virulent *Bp* strains, giving more time for the development of a humoral response. We did not collect information on the duration of symptoms at presentation for this cohort, but there was no difference in mortality between people with and without diabetes in this cohort.<sup>28</sup> An alternative or additional reason may be chronic hyperactivation of the innate immune response in type 2 diabetes resulting in polyclonal B-cell stimulation and enhanced antibody production to stimulus.<sup>29</sup> This was the suggested explanation for a study finding higher hemagglutination inhibition and ELISA antibody responses to influenza vaccine seen in elderly diabetic people compared with elderly non-diabetic people.<sup>30</sup> A further possible factor is medications taken for diabetes such as metformin and glibenclamide having an impact on immune responsiveness to melioidosis,<sup>31</sup> although no relationship was seen between medication and IHA titer in this study. Ongoing work in our laboratory is exploring the mechanisms of enhanced B-cell responsiveness in people with diabetes.

We also found that *Bp*-seronegative status (IHA titer  $< 1:80$ ) was significantly associated with high circulating neutrophil counts ( $\geq 12,000$  cells/ $\mu$ L). Neutrophilia due to a marked increase in bone marrow production of neutrophils and massive recruitment of immature neutrophils into the circulation is a hallmark of sepsis.<sup>32,33</sup> All classes of blood cells are derived from hematopoietic stem cells in the bone marrow, and a “myeloid left shift” toward increased production of neutrophils in the bone marrow can lead to a reduction of progenitor cells for lymphocyte production. An additional relationship between neutrophils and antibody levels is that in health, neutrophils in the perifollicular area of the spleen play a B-cell helper role in stimulating antibody production from marginal zone B cells.<sup>34,35</sup> Immature neutrophils have decreased chemotactic activity compared with mature neutrophils<sup>32,36</sup> which may be due to decreased expression of chemotaxis receptors such as the IL-8 receptor B (CXCR2)<sup>32</sup> and decreased deformability.<sup>36,37</sup> Thus, patients with neutrophilia during acute sepsis may have a predominance of immature neutrophils that are less able to support B-cell function. Nevertheless, the mechanistic correlation between neutrophil count and antibody titer in melioidosis requires further characterization.

The IHA test remains reliable as a serological survey for evidence of exposure in healthy populations<sup>38–41</sup> and for evaluating non-endemic inhabitants without significant immunocompromise returning from endemic regions with symptoms suggestive of melioidosis. However, using IHA for serodiagnosis of acute melioidosis patients living in areas of endemicity is discouraged. One major hindrance of interpretation of IHA results in endemic areas is the presence of high background IHA titers, which may lead to false-positive diagnosis.<sup>5,7,9</sup> We detected 28% of healthy controls having seropositive IHA titers at an elevated cutoff titer of 1:160 as suggested from the previous study.<sup>7</sup> However, the seroprevalence decreased from 72.5% (cutoff  $\geq 1:80$ ) to 60% (cutoff  $\geq 1:160$ ) in culture-proven patients at this cutoff titer. Our results indicate that increasing

the cutoff IHA titer for diagnosis to  $\geq 1:160$  would not yield adequate sensitivity and specificity as a test in hyperendemic areas.

Another issue with the IHA test is the existence of undetectable IHA titers in acute patients with culture-proven melioidosis, as found in our longitudinal study and in the Australian studies.<sup>6,11</sup> Persistent nonreactive IHA results 12 weeks post illness show that this was not due to delayed antibody responses. The IHA-nonreactive sera in the Australian study remained negative to autologous bacterial antigens in an IHA retest, indicating that the negative IHA titer was regardless of the bacterial isolates used in the assay.<sup>42</sup> Both studies show that IHA seropositivity during the acute stage was lower in patients aged older than 45 years. This may be due to age-related decline of naive B-cell production and impaired memory B cells, leading to a decrease of circulating antibodies<sup>26,43,44</sup> and their functional activities.<sup>45</sup> However, it is also possible that patients aged 45 years or older may do less rice farming work and therefore have lower exposure to the bacteria including avirulent *Burkholderia* species.

Variation in *Bp* antigen preparation for the IHA test between different studies hinders the standardization of the assay. Most published studies have used bacterial strains local to the population tested, but some studies used bacterial isolates from neighboring countries<sup>8,22</sup> or from another continent.<sup>46</sup> The variation in antigen preparation also includes the number of strains used in the test, ranging from one<sup>47,48</sup> to 25 strains,<sup>9,49</sup> and the incubation period of bacterial culture during preparation, ranging from 3 days<sup>23</sup> to 2<sup>14,47</sup> to 3 weeks.<sup>49</sup> Several studies reported a comparison between IHA and alternative serodiagnostic tests including complement fixation,<sup>14,50</sup> the indirect fluorescence assay,<sup>51</sup> dot blot immunoassay,<sup>52</sup> immunochromatographic test,<sup>53</sup> and ELISA.<sup>11,48,52,54</sup> Among antibody measurement approaches, ELISA using crude whole bacteria<sup>10,48</sup> or purified *Bp* antigens such as O-polysaccharide<sup>55</sup> and type VI secretion system HCP protein<sup>56,57</sup> appears to be the most promising method in our opinion, with improved sensitivity and specificity. Enzyme-linked immunosorbent assay has the advantage of allowing focus on IgG subclasses and use of reader machines to reduce interlaboratory variation. Other approaches based on pathogen detection strategy including PCR,<sup>58–60</sup> microarray,<sup>61,62</sup> lateral flow immunoassay<sup>63–66</sup> and matrix-assisted laser desorption ionization-time-of-flight mass spectrometry<sup>67,68</sup> are also in development to confirm melioidosis diagnosis.

The lack of significant association between a positive IHA result and protection against fatal melioidosis when possible confounding factors were added to the model suggests that antibody quantity alone is insufficient to drive protective humoral immunity. An understanding of the biological functions of protective antibodies to *Bp* infection is essential for melioidosis vaccine development. Therefore, we are presently developing assays to dissect the biological abilities of antibodies in protection against melioidosis.

In conclusion, results from a new unique dataset of an adult healthy population living in a melioidosis-endemic region highlight the limitation of IHA for diagnosis of acute melioidosis in this setting, as around half of healthy people were seropositive. We have unequivocally demonstrated the strong relationship between seropositivity in adults in the region and both occupation as a rice farmer and residence in rural areas. We also demonstrated the varying IHA seropositive pattern among patients with culture-confirmed melioidosis, and a

poor sensitivity of IHA in patients with bacteremia. Our findings emphasize the importance of developing new serodiagnostic approaches for melioidosis with improved specificity and sensitivity, alongside characterizing the cross-reactivity and functional properties of antibodies that are essential for successful management of melioidosis.

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