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INDIRECT HEMAGGLUTINATION ASSAY IN PATIENTS WITH MELIOIDOSIS IN NORTHERN AUSTRALIA

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Abstract. Melioidosis is caused by the saprophytic organism *Burkholderia pseudomallei*. The use of the indirect hemagglutination assay (IHA) has found widespread use in areas endemic for this disease. Using this assay, we explored the serologic profile of 275 patients with culture-confirmed melioidosis in the Northern Territory of Australia. Based on a threshold titer of 1:40, the sensitivity of the IHA on admission was 56%. Female patients, those with positive blood cultures, and those with pneumonia independently predicted a negative IHA result. Most patients (68%) with negative admission IHA titers subsequently seroconverted. Most patients (92%) with positive admission IHA titers had persistently positive IHA titers. Relapses were not observed in 36 patients who had a negative IHA at least 1 month after admission, irrespective of initial admission IHA. The IHA has limited utility as a diagnostic test for acute disease, and most patients subsequently have persistently positive titers after recovery from illness.

INTRODUCTION

Melioidosis, which is caused by the saprophytic gram-negative bacillus *Burkholderia pseudomallei*, is endemic in northern Australia and southeast Asia.[1] Reported annual incidence rates of this disease vary from 16.5 cases per 100,000 population in northern Australia[2] to 4.4 per 100,000 in northeastern Thailand.[3] It has protean presentations, may affect almost any site in the body, and has a spectrum of severity from acute fulminant sepsis to chronic infection. Risk factors for disease include diabetes, heavy alcohol use, and chronic renal disease.[4] Diagnosis relies on positive culture; rapid diagnostic tests, particularly based on the detection of antibodies, are confounded by the high prevalence of positive serologic results in disease-endemic areas[2,5–7]

The immune mechanisms that mediate resistance to melioidosis are poorly defined. Repeated natural exposure to *B. pseudomallei* and antigenically related saprophytic organisms are sufficient to induce specific antibodies. Although some studies have suggested that high levels of specific antibodies may be protective,[8,9] specific antibodies in most patients are insufficient to prevent or clear infection with *B. pseudomallei*. Other work has demonstrated that a specific cell-mediated response is present in survivors of melioidosis,[10] but the lack of association of melioidosis with infection with human immunodeficiency virus suggests that other mechanisms may mediate protection against *B. pseudomallei*.

The indirect hemagglutination assay (IHA) remains the only widely used serologic test for melioidosis, despite its known poor sensitivity and specificity.[7,11–14] In this study, we explore the serologic profile of patients with culture-confirmed melioidosis in northern Australia.

METHODS

Data on patients presenting to the Royal Darwin Hospital since October 1989 have been collected prospectively in the Darwin Prospective Study. The catchment area of the hospital incorporates the Top End region of the Northern Territory, encompassing an area of more than 500,000 km^2 and approximately 150,000 people. The data collected include clinical and demographic data, as well as the results of investigations and follow-up data.

The IHA was conducted as previously described[15,16] using antigen produced from a combination of three clinical isolates of *B. pseudomallei* previously isolated from different geographic regions in the Top End of the Northern Territory. A negative serologic result was defined as an IHA titer $\leq$ 1:20, low positive as 1:40 or 1:80, high positive as between 1:160 and 1:1,280 inclusive, and very high positive as > 1:1,280. Patient serum for testing was heat inactivated at 56°C and treated with unsensitized ovine erythrocytes. After centrifugation, the supernatant was then serially titrated and reacted with ovine erythrocytes sensitized to *B. pseudomallei* antigen. Unsensitized ovine erythrocytes were used as a control.

We defined admission serologic results as the IHA titer obtained within three days of admission to a hospital. Relapse was defined as the recurrence of the clinical features of melioidosis after apparent cure from a previous episode of melioidosis with appropriate antibiotic therapy. Previous local work has defined relapse to be due to the same strain in 93% of the cases.[17] Chronic disease was defined as occurring in patients with more than two months of symptoms.[17] Severe sepsis was defined according to established criteria that include the presence of sepsis and end-organ dysfunction.[18]

We identified four patterns of serologic change in patients with melioidosis; those with negative IHA titers on admission but who subsequently seroconverted (seroconversion), those with positive IHA titers on admission but whose IHA titer waned to negative subsequently (seroreversion), those with persistently negative IHA titers, and those with persistently positive titers. We specifically considered IHA in three clinically distinct groups of interest: patients with chronic melioidosis, patients with later relapsed disease and patients with severe melioidosis (indicated by the presence of bacteremia and/or severe sepsis).

Proportions across ordered groups were compared using Cuzick's non-parametric test for trend. Proportions across non-ordered groups were compared using the Fisher's exact

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test. Geometric means of IHA titers of groups were compared using the log rank test. Multivariate models were constructed using backward stepwise selection examining clinical factors that predicted IHA titer. The IHA titers were considered both in ordered categories (negative, low positive, positive, and highly positive) and as a dichotomous variable (positive or negative). Post-regression diagnostic plots were constructed to discount the effect of statistical outliers. Statistical tests were performed using Intercooled Stata version 8.2 (Stata Corporation, College Station, TX). Ethical approval for this review was obtained from the Human Research Ethics Committee of the Menzies School of Health Research and the Department of Health and Community Services.

RESULTS

During the period from October 1989 to September 2002, 344 patients presented with culture-confirmed melioidosis. Of these patients, the results of IHA on admission were available for 275 patients and were included in this study. Of these patients, 201 (73%) patients had IHA titers determined at least one month subsequent to admission and were included in the longitudinal analysis.

Of the 275 patients in this study, the median age at first admission was 49 years (including 12 pediatric patients < 18 years of age) and 203 (74%) were male. Sites of infection were similar to that previous described[4] and included 140 (51%) patients with pneumonia, 42 (15%) with genitourinary disease, 34 (12%) with sepsis without a clinical focus, 39 (14%) with infections involving skin or soft tissue, 11 (4.0%) with encephalomyelitis or central nervous system infection, and 9 (3.2%) involving other sites. Risk factors included diabetes in 107 patients (39%), alcoholism in 97 patients (35%) and chronic renal disease in 24 patients (8.7%). Severe sepsis was present on first admission in 56 (21%) of the 272 patients where this was recorded and bacteremia in 135 (51%) patients of the 273 patients where blood cultures were performed. A chronic presentation was noted in 32 (12%) patients. Culture-confirmed relapse was seen in 27 patients. The overall mortality was 14% (n = 38).

Admission IHA titers. A significant proportion of patients had negative IHA titers (44%, n = 120) on admission. Of the 155 patients (56%) with positive serologic results, 42 (27%) had low positive titers, 77 (50%) had high positive titers, and 36 (23%) had very high positive titers. Clinical characteristics and admission IHA titers are shown in Table 1. The sensitivity of the IHA was 56% at a threshold titer of 1:40 and 41% at a threshold titer of 1:160. Of the 32 patients with chronic presentations of melioidosis, the sensitivity of the IHA was 81% and it was higher than in patients with acute presentations (53%; $P = 0.002$, by Fisher's exact test).

Univariate analysis showed that female sex, pneumonia, acute presentation, positive blood cultures, and chronic renal disease predicted a negative IHA titer. Multivariate analysis showed that female sex, pneumonia, and positive blood cultures were independently predictive of negative IHA titers.

TABLE 1
Clinical factors and admission indirect hemagglutination assay titer in 275 patients

Variable	No. of patients	Negative	Low positive	High positive	Very high positive	Significance* *P*	Adjusted coefficient†	Significance *P*
All patients	275	120 (44%)	42 (15%)	77 (28%)	36 (13%)			
Age, years								
< 49	135	55 (41%)	17 (13%)	40 (30%)	23 (17%)	0.08		
> 49	140	65 (46%)	25 (18%)	37 (26%)	13 (9.3%)			
Sex								
Male	203	78 (38%)	33 (16%)	62 (31%)	30 (15%)	0.004	–0.34	0.02
Female	72	42 (58%)	9 (13%)	15 (21%)	6 (8.3%)			
Ethnicity								
Aboriginal	147	70 (48%)	21 (14%)	39 (27%)	17 (12%)	0.16		
Non-aboriginal	128	50 (39%)	21 (16%)	38 (30%)	19 (15%)			
Diabetes								
Present	107	51 (48%)	16 (15%)	30 (28%)	10 (9.4%)	0.18		
Absent	168	69 (41%)	26 (15%)	47 (28%)	26 (15%)			
Heavy alcohol intake								
Present	97	43 (44%)	13 (13%)	31 (32%)	10 (10%)	0.82		
Absent	178	77 (43%)	29 (16%)	46 (26%)	26 (15%)			
Chronic renal failure								
Present	24	16 (67%)	1 (4.2%)	5 (21%)	2 (8.3%)	0.052		
Absent	251	104 (41%)	41 (16%)	72 (29%)	34 (14%)			
Pneumonia								
Present	140	78 (56%)	17 (12%)	32 (23%)	13 (9.3%)	< 0.001	–0.42	0.001
Absent	135	42 (31%)	25 (19%)	45 (33%)	23 (17%)			
Presentation								
Acute	243	114 (47%)	36 (15%)	65 (27%)	28 (12%)	0.001	–0.44	0.035
Chronic	32	6 (19%)	6 (19%)	12 (38%)	8 (25%)			
Severe sepsis‡								
Present	56	30 (54%)	4 (7.1%)	15 (27%)	7 (13%)	0.32		
Absent	216	89 (41%)	38 (18%)	62 (29%)	27 (13%)			
Blood cultures‡								
Positive	138	78 (57%)	17 (12%)	30 (22%)	13 (9.4%)	< 0.001	–0.41	0.002
Negative	135	40 (30%)	25 (19%)	25 (19%)	23 (17%)			

* Proportions across ordered groups were compared using Cuzick's non-parametric test for trend.
† Final model was selected by backward stepwise selection; candidate independent factors were selected using $P < 0.2$.
‡ Presence of severe sepsis was not documented in three patients; blood cultures were not performed in two patients.

Table 1 lists factors associated with a higher category of IHA titer; with the exception of chronic renal failure (univariate $P = 0.053$) and acute presentation (multivariate $P = 0.10$), these factors were similar to those associated with a positive titer. The multivariate model was robust on exclusion of 11 outliers identified by a leverage versus squared residual plot, but only accounted for 14% of the variation in admission IHA titer category observed (r^2).

The distribution of IHA titers was not significantly different in the 38 patients that died of melioidosis, with 22 (58%) having negative admission titers, 3 (7.9%) low positive titers, 9 (24%) high positive titers, and 4 (11%) very high titers ($P = 0.13$, by non-parametric test for trend compared with survivors). A higher proportion of patients that died had negative admission IHA titers but this was not statistically significant (58% versus 41%; $P = 0.078$, by Fisher's exact test).

Longitudinal IHA titers. Of the 275 patients in this study, 201 had subsequent IHA titers at least one month after admission and were further analyzed. Of the 82 patients with initial negative IHA titers, 56 (68%) seroconverted and 26 (32%) remained persistently negative by IHA. Of the 119 patients with initial positive IHA titer, 10 (8%) had subsequent negative IHA titers and 109 (92%) remained persistently positive. Details of patterns of serologic changes and clinical factors are shown in Table 2.

Patients with chronic disease had persistently positive IHA titers in 84% of the cases, a pattern only observed in 50% of the 176 patients with acute presentations where a subsequent IHA was conducted. Patients with positive blood cultures had a seroconversion pattern in 41% of cases where a repeat IHA was available (a pattern observed in 17% of patients that were blood culture negative). In contrast, persistently positive IHA titers were observed in 67% of patients with negative cultures where repeat IHA results were available compared with 40% of blood culture–positive cases. There were no other significant correlations observed between clinical features and patterns of serologic changes.

Relapsed disease. Of the 275 patients in this study, 27 (10%) patients had 33 episodes of relapse (including six with two episodes of relapse). First relapses occurred a median of 27 weeks after the first presentation (interquartile range [IQR] = 13–36 weeks) and second relapses occurred a median of 87 weeks after first presentation (IQR = 6–115 weeks). Admission titers on relapse were available in 29 (87%) of 33 episodes; 4 patients had negative IHA titers, 6 patients had low positive titers, 10 patients had high positive titers, and 9 patients had very high positive titers.

Of the 27 patients with relapse, 19 had IHA titers determined between first and second episodes. A seroconversion pattern occurred after the first presentation in 5 patients (representing 9% of the 56 patients where this pattern was observed), and a persistently positive pattern was observed in 14 patients (13% of the 109 patients who had persistently positive serologic results). None of the 36 patients with negative

TABLE 2

Longitudinal patterns of indirect hemagglutination assay (IHA) titers and clinical profiles in 201 patients where follow-up IHA results were available

Variable	No. of patients	Seroconversion	Persistent negative	Seroreversion	Persistent positive	Significance* *P*
All patients	201	56 (28%)	26 (13%)	10 (5%)	109 (54%)	
Age, years						
< 47	95	24 (25%)	11 (12%)	4 (4%)	56 (59%)	0.63
> 47	109	32 (30%)	15 (14%)	6 (6%)	53 (50%)	
Sex						
Male	156	41 (26%)	19 (12%)	7 (4%)	89 (57%)	0.44
Female	45	15 (33%)	7 (16%)	3 (7%)	20 (44%)	
Ethnicity						
Aboriginal	101	33 (33%)	13 (13%)	5 (5%)	50 (50%)	0.46
Non-aboriginal	100	23 (23%)	13 (13%)	5 (5%)	59 (59%)	
Diabetes						
Present	82	26 (32%)	10 (12%)	3 (4%)	43 (52%)	0.73
Absent	119	30 (25%)	16 (13%)	7 (6%)	66 (55%)	
Heavy alcohol intake						
Present	73	25 (34%)	7 (10%)	2 (3%)	39 (53%)	0.31
Absent	128	31 (24%)	19 (15%)	8 (6%)	70 (54%)	
Chronic renal failure						
Present	14	5 (26%)	4 (29%)	1 (7%)	4 (29%)	0.10
Absent	187	51 (27%)	22 (12%)	9 (5%)	105 (56%)	
Pneumonia						
Present	96	37 (39%)	13 (14%)	6 (6%)	42 (43%)	0.008
Absent	105	19 (18%)	13 (12%)	4 (4%)	67 (64%)	
Presentation						
Acute	176	55 (31%)	23 (13%)	10 (6%)	88 (50%)	0.004
Chronic	25	1 (4%)	3 (12%)	0	21 (84%)	
Severe sepsis†						
Present	179	10 (48%)	2 (10%)	1 (5%)	8 (38%)	0.22
Absent	21	46 (25%)	24 (13%)	9 (5%)	100 (56%)	
Blood cultures†						
Positive	92	38 (41%)	14 (15%)	3 (3%)	37 (40%)	< 0.001
Negative	108	18 (17%)	11 (10%)	7 (6%)	72 (67%)	

* Proportions across non-ordered groups were compared using the Fishers's exact test.
† Presence of severe sepsis was not documented in one patient; blood cultures were not performed in one patient.

interval IHA titers relapsed, irrespective of their admission IHA titer. Three patients with relapse had persistent titers > 1:1,280 (a pattern observed in 9 patients in this study) and one additional patient had a titer > 1:1,280 after a negative titer on first admission.

DISCUSSION

The IHA is a cheap and simple test that is the only serologic test with widespread use in the diagnosis of melioidosis in disease-endemic countries. It has remained essentially unchanged since it was first described in 1965.[19] However, previous studies have demonstrated a poor specificity due to a high background rate of positive test results in disease-endemic populations, as well as poor sensitivity.[7,11–14] Our study suggests that the sensitivity of admission IHA and the subsequent patterns of change vary with the characteristics of melioidosis. Whether acute disease is the result of a poor humoral response or occurs prior to an effective response is not clear.

We classified IHA by criteria in common use; in Australia, a titer of 1:40 is considered positive,[5] and in Thailand, a titer of 1:160 is considered positive.[20] In Queensland, Australia, Ashdown and Guard reported that the background rate of seropositivity in Australian patients based on the complement fixation test was lower than in southeast Asian patients.[5] In two recent studies, the rate of positive serologic results in controls without melioidosis was 8.7% (based on a threshold IHA titer of 1:40), but in Thailand a rate of 35% was observed (based on a higher IHA titer of 1:160).[7,14] The reason for this difference is not clear, but recent work has suggested that this is not due to cross-reactivity to the antigenically related *B. thailandensis*.[21] We speculate that this represents a high exposure to *B. pseudomallei* in the Thai population.

A limitation of the IHA is the variation in the strains used (typically a combination of local clinical strains) and the uncharacterized nature of the antigenic epitopes and antibodies involved. Based on fractionation of the antibodies detected in positive IHA sera in 14 patients, Ashdown suggested that the IHA reflected the IgM levels.[22] With the lack of cross-reactivity to *B. thailandensis*, it appears that lipopolysaccharide (conserved between the species) is not the major antigenic epitope in the IHA, but rather that other antigens, such as capsular polysaccharide, are more important in this assay.[21] Whether strain variation between Thailand and Australia is an important determinant of IHA titer has not been explored. Studies using more sophisticated assays have defined that a broad humoral response, involving all classes of immunglobulins, is present in melioidosis, but the reasons for the poor efficiency of these antibodies in preventing or clearing *B. pseudomallei* is not known.[23]

Few studies have examined the pattern of serologic change after an episode of melioidosis; in seven patients, a decrease in the IgG level (detected by enzyme-linked immunosorbent assay) appeared to correlate with successful treatment, but only one patient in that study had a culture-confirmed relapse.[24] In our study, the most common pattern of serologic change was persistently positive IHA titers after a positive admission IHA.

Although we are not able to determine the longer term significance of positive titers, few patients with persistently positive titers were observed to relapse during the study period, suggesting that persisting positive serologic results does not necessarily mean persisting presence of infection. Latent infection with subsequent disease has been described for periods of up to 62 years after presumed exposure.[25] Positive IHA results may not necessarily represent latent infection. Although it was estimated that up to 225,000 returned U.S. servicemen had positive serologic resulting after the Vietnam War,[26] the feared Vietnamese time bomb failed to materialize.[27] In this study, one-third of the patients with persistent titers > 1:1,280 were observed to relapse, although the small numbers precluded definite conclusions. Although we did not systematically examine pre-admission IHA titers, long-term follow-up of patients with high IHA titers but no initial evidence of disease at our center does occasionally unmask subsequent melioidosis caused by reactivation from a presumed latent focus.[28] Conversely, it was observed that patients with negative post-admission IHA titers (either after initial positive or negative IHA titers on first admission) did not subsequently relapse.

The IHA and other serologic tests lack both sensitivity and specificity. More work is required to identify patients with melioidosis early in the course of illness and to confirm our observation that patients with a negative IHA result after treatment have a low rate of relapse.

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