Original Article

The Use of Indirect Immune-fluorescence Antibody Testing (IFAT) IgM And IgG In the Diagnosis of Melioidosis

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Abstract

Introduction: Meliodosis is an important public health disease caused by*Burkholderiapseudomallei*. Early laboratory diagnosis is crucial for appropriate treatment due to its high mortality rate. ***Objective:*** This study is conducted to assess the potential role of the in-house IFAT IgM and IgG as the serodiagnostic tool in melioidosis and to determine the cut-off levels. ***Method:*** 40 culture-confirmed melioidosis patients were recruited. Controls consisted of a group of 40 patients without active infection and another group of 40 patients with positive blood culture for organisms other than*Burkholderiapseudomallei*. ***Results and Discussion:*** Using the receiver operating characteristic (ROC) curve, the best cut-off levels determined to diagnose melioidosis are 1:20 for IgM and 1:80 for IgG. Of these cut off levels, the sensitivity and specificity for IgM are 72.5% and 80% respectively and 65% and 87.5%respectively for IgG which also has high background seropositivity. ***Conclusion:*** IFAT IgM at the cut-off level 1:20 is recommended for diagnosis.

Keywords: Melioidosis, Indirect Immunofluorescent Antibody Test, Cut-off.

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Introduction

It has been ten decades since the melioidosis outbreak in Pahang with the consequent 8 fatalities[1] and meliodosis remains as the potential fatal endemic infectious disease in the Southeast Asia and Northern Australia.[2] Direct exposure to contaminated soil and surface water is a well-known transmission mode of this deadly disease caused by *Burkholderiapseudomallei* (*B. pseudomallei).*[3,4]*B. pseudomallei*is a facultative intracellular Gram-negative rod that is able to grow on the routinely used microbial media such as Blood agar, MacConkey and Nutrient agar upon incubation at 35 to 37ºC. Thus, conventional culture method still remains the gold standard for definitive diagnosis of melioidosis despite its poor sensitivity (60.2%).[5] However, the culture result is only available after 3 to 5 days and hence the resultant delay in administering appropriate

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treatment to the infected patients.[6] In the meantime, its diverse clinical manifestations pose further challenges in the clinical diagnosis rendering further difficulty in instituting the treatment.[7]In some cases, the bacterium is not always isolated[2] and this may cause further dilemma to clinicians in deciding on the continuation of prolonged maintenance therapy in some patients who show good responses to the initial, empirical therapy.

Early detection of the causative agent is life-saving especially in septicaemic patients. Therefore, serological tests are often employed for a rapid diagnosis of meliodosis. These antigen tests are performed directly on the clinical specimens such as serum, urine and sputum in which results are available within a day.[8] Indirect Hemagglutination Assay (IHA) test has been widely used in endemic regions such as Northern Australia and Northeast Thailand.[9,10] This assay is developed and established by the "in house" protocol and generally has poor sensitivity and specificity due to the weak immunogenicity of antigens used in its preparation.[11]IHA testing is not encouraged to be used as a diagnostic tool in an endemic area due to high seropositivity in healthy subjects who are likely repetitively exposed to *B. pseudomallei*.[12] Meanwhile, ELISA testing is yet to be recognized as a reliable serodiagnostic tool as to identify the perfect antigen(s) to be used in this method.[13]

On the contrary, Indirect Fluorescent Antibody Testing (IFAT) has been used in Malaysia for many years since it was first described by Vadivelu et al. in 1995.[14] In comparison to IHA, this assay is able to give specific antibody titers of individual immunoglobulin M (IgM) or immunoglobulin G (IgG) or both. Currently, IFAT IgM test for samples from hospitals all over the country at the Institute of Medical Research (IMR). It is used concomitantly with the serological culture for the better diagnostic yield in melioidosis. However, this test has not yet been well-validated in any prospective clinical trial. Hence, the objective of this current study is to evaluate the potential role and efficacy of the in-house IgM and IgG IFAT methods in the diagnosis of melioidosis and to determine the diagnostic cut off levels among Malaysian patients.

Materials and Methods

Bacterial Strains

The strain of *B. pseudomallei* used in IFAT was obtained from the blood culture of a patient at Hospital Tengku AmpuanAfzan (HTAA), Kuantan, Pahang. This strain was identified using Francis Medium,[15] conventional biochemical assimilation tests and API 20NE System (Bio-Merieux, France). A bacterial suspension was prepared from pure colonies in Trypticase Soy Broth (TSB) which was heat-killed before being used as the antigen in IFAT.

Melioidosis patients and controls

This study was conducted using sera collected from November 2014 to November 2015. A total of 120 patients were recruited in this study. 40 of them were from culture-confirmed melioidosis cases (28 of them were from HTAA and the remaining 12 patients were from Hospital Sultanah Nur Zahirah (HSNZ), Kuala Terengganu, Terengganu). 80 patients were recruited as control subjects consisting of 40 consecutively-selected patients with positive blood culture for bacteria other than *B. pseudomallei* and another 40 patients were healthy subjects (without any clinically evident infection) who came for routine blood investigation during their hypertension or diabetes clinics. Sample collection for melioidosis patients was done on day 1 (±2days) of the culture-positive results.

Indirect Immunofluorescent Antibody Testing (IFAT)

The IFAT was carried out as described by Ashdown[16]with modifications. Briefly, the bacterial antigen was washed and resuspended in phosphate-buffered saline (PBS), pH 8.5. Then a working antigen was prepared using PBS (pH 7.3) and coated onto 12-wells of Teflon coated slides before being air-dried and fixed with cold acetone. Patients' sera were serially diluted two-folds in PBS (pH 7.3) starting from 1:10 until 1:160, then each dilution overlaid onto the antigen wells and incubated at 37 ºC for 30 minutes in a moist chamber. A fluorescein isothiocynate (FITC)-tagged anti-human globulin IgM and IgG specific dye (Kirkegaard& Perry Laboratories (KPL), United States) was each separately added after washing the slides with PBS (pH 7.3) for three times and subsequently incubated for further 30 minutes in a moist chamber at 37 ºC. The slides were then washed with PBS (pH 7.3) and mounted using buffered glycerol. Positive and negative sera were included in each batch of tests as controls. Finally, the stained slides were examined under a fluorescent microscope at 40X magnification.

A positive result was determined by appearance of apple green fluorescence of the bacilli of *B. pseudomallei*. If high positive cell counts were noted at dilution 1:160, testing at further higher dilutions of 1:320, 1:640 and 1:1280 was performed. Meanwhile, if a negative result was noted at dilution 1:10, it would be recorded as <10.

Statistical Analysis

Demographic data of the patients with melioidosis and subjects in the control groups were compared using one-way ANOVA for age and Chi-square for gender and diabetic status. In order to perform valid statistical analysis on the IgM and IgG levels, means of antibody titers in each immunoglobulin class were log-transformed and expressed as geometrical means (GM) and standard deviations as geometrical standard deviations (GSD).[17]For this purpose, titers of <10 were presumed as equal to 5 to avoid any missing value during the statistical analysis.

The optimum cut-off value for IFAT-IgM and IFAT-IgG was determined by using Receiver Operating Characteristics (ROC) curve. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated. Then, the seropositivity rate for specific IgM and IgG antibody between melioidosis and non-melioidosis (control groups) was compared by Chi-square method. All data obtained in this study were analyzed using SPSS Version 21 for windows. Data with p values of <0.05 were considered as statistically significant.

Results

All 40 patients with melioidosis in this study are culture-positive for blood (40). 9 of them are also positive for pus/tissue (5), sputum (3) and knee joint aspirate (1) cultures. Majority of the cases are newly diagnosed (38) and only 2 are re-infections. As for the control group, 18 of them have positive cultures for gram-positive bacteria while 22 are culture-positive for gram-negative bacteria. They are no difference in the mean age and ethnic group distribution for both melioidosis patients (M) and the control groups (Table 1). However, we observe that there are significantly more diabetic patients

Table 1. Demographic information of the patients

	M		Control Subject Group(s)				Overall Percentage	p-value
			Cp		Ca			
	n	%	n	%	n	%		
Age in years								
20-40	6	15.0	8	20.0	3	7.5	14.2	
41-60	22	55.0	14	35.0	24	60.0	50.0	
>60	12	30.0	18	45.0	13	32.5	35.8	
Mean Age*	53 (12)		58 (17)		56 (12)			0.297[a]
Ethnicity								
Malay	36	90.0	33	82.5	27	67.5	80.0	
Chinese	2	5.0	5	12.5	8	20.0	12.5	
Indian	1	2.5	2	5.0	4	10.0	5.8	
Others	1	2.5	0	0.0	1	2.5	1.7	
Gender								
Male	34	85.0	27	67.5	22	55.0	69.2	0.014[b]
Female	6	15	13	32.5	18	45.0	30.8	
Diabetic status								
Yes	37	92.5	25	62.5	19	47.5	67.5	0.0007[b]
No	3	7.5	15	37.5	21	52.5	32.5	

M= Patients with melioidosis, Cp= patients with other bacterial infections, Ca= out-patients without apparent infection, * value represent mean (standard deviation), [a] = p value by one-way ANOVA, [b] = p value by Chi-square test and n= number of patients.

in melioidosis group compared to the controls (92.5% in melioidosis group versus 62.5% in Cp (other infections) group versus 47.5% in Ca (no infections) group, p=0.0007).

IFAT-IgM and IFAT-IgG antibody titers are compared among all groups as shown in Table 2. There is significant difference observed in the geometrical means (GM) among the groups for both IgM and IgG antibody titers (IgM, F(2,117) = 31.179, p= 0.0005 and IgG, F(2,117) = 28.948, p= 0.005). The Scheffe post-hoc test reveals that group M is statistically different when compared to the Cp and Ca groups (p= 0.0005) but there is no significant difference between the two control groups, Cp and Ca (p= 0.680). Therefore, for further analysis, Cp and Ca groups are combined into one group (C). There is 22.5% of melioidosis patients with IgM titer of <1:10 as compared to 65% in control (p<0.001). Similar trend is also observed for IgG, whereby 17.5% of melioidosis patients have IgG of <1:10 as compared to 58.7% of control (p<0.001).

Table 2. Distribution of specific IgM and IgG antibody titers among all study groups

Group(s)	IgM Titers						
	<10[c] n(%)	**10 n(%)**	**20 n(%)**	**40 n(%)**	**80 n(%)**	**160 n(%)**	**320 n(%)**
M	9 (22.5)	2 (5.0)	4 (10.0)	1 (2.5)	12 (30.0)	6 (15.0)	6 (15.0)
Cp	27 (67.5)	9 (22.5)	2 (5.0)	0 (0.0)	2 (5.0)	0 (0.0)	0 (0.0)
Ca	25 (62.5)	3 (7.5)	8 (20.0)	1 (2.5)	3 (7.5)	0 (0.0)	0 (0.0)

Group(s)	IgGTiters							
	<10[d] n(%)	**10 n(%)**	**20 n(%)**	**40 n(%)**	**80 n(%)**	**160 n(%)**	**320 n(%)**	**640 n(%)**
M	7 (17.5)	3 (7.5)	3 (7.5)	1 (2.5)	13 (32.5)	5 (12.5)	5 (12.5)	3 (7.5)
Cp	24 (60.0)	7 (17.5)	1 (2.5)	1 (2.5)	6 (5.0)	1 (2.5)	0 (0.0)	0 (0.0)
Ca	23 (57.5)	6 (15.0)	6 (15.0)	2 (5.0)	3 (7.5)	0 (0.0)	0 (0.0)	0 (0.0)

M = Patients with melioidosis, **Cp**= patients with other bacterial infection, **Ca**= out-patients without any infection, n= value represents number of patients and [c, d] = p value by Chi-square test (c = 0.001 and d = 0.001).

The Receiver Operating Characteristic (ROC) curve for IFAT-IgM (Figure 1) yields the AUC (Area Under the Curve) value of 0.810 (95% CI: 0.72-0.90, p<0.001) and AUC value of 0.809 (95% CI: 0.72-0.90, p<0.001) for IFAT-IgG (Figure 2). The optimal cut-off points for IFAT-IgM and IFAT-IgG are determined as 1:20 and 1:80 respectively. The sensitivity and specificity of IFAT-IgM at 1:20 titer are 72.5% and 80.0% respectively with positive and negative predictive values of 64.4% and 85.3% respectively (Table 3). Meanwhile, the sensitivity and specificity of IFAT IgG at 1:80 titer are 65.0% and 87.5% respectively with positive and negative predictive values of 72.2% and 83.3% respectively (Table 3).

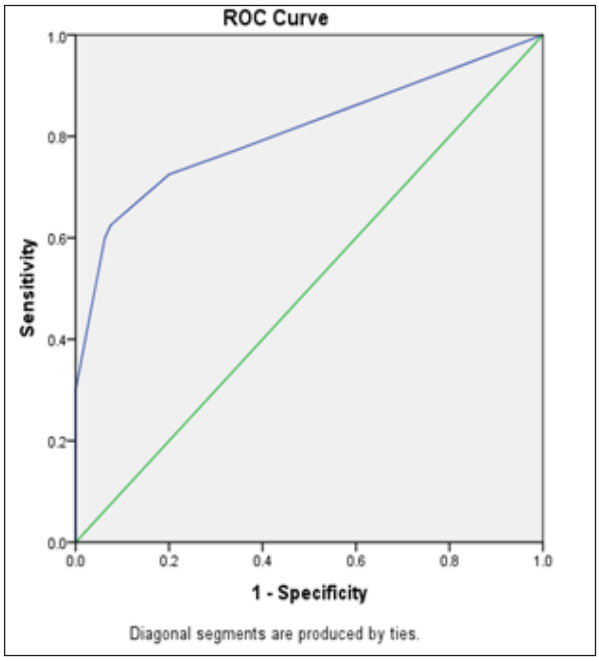

Figure 1: ROC curve for IFAT-IgM

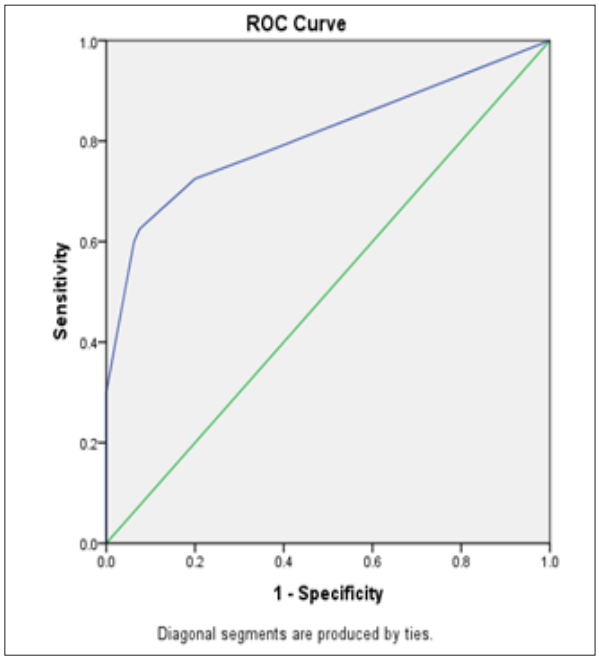

Figure 2: ROC curve for IFAT-IgG

Table 3. Sensitivity, Specificity, Positive and Negative Predictive Value (PPV and NPV) at Different Cut-off Pointsof IFAT-IgM and IFAT-IgG

IFAT-IgM								
Titer (dilution)	M (n=40)		C (n=80)		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	TP	FN	FP	TN				
<10	40	0	80	0	100	0.0	33.3	0.0
10	31	9	13	27	77.5	65.0	52.5	85.2
20	29	11	16	64	72.5	80.0	64.4	85.3
40	25	15	6	74	62.5	92.5	80.6	83.1
80	24	16	5	75	60.0	93.8	82.8	82.4
160	12	28	0	80	30.0	100	100	74.1
320	6	34	0	80	15.0	100	100	70.2

IFAT-IgG								
Titer (dilution)	M (n=40)		C (n=80)		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	TP	FN	FP	TN				
<10	40	0	80	0	100	0.0	33.3	0.0
10	33	7	33	47	82.5	58.8	50.0	87.0
20	30	10	20	60	75.0	75.0	60.0	85.7
40	27	13	13	67	67.5	83.8	67.5	83.8
80	26	14	10	70	65.0	87.5	72.2	83.3

IFAT-IgG								
Titer (dilution)	M (n=40)		C (n=80)		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	TP	FN	FP	TN				
160	13	27	1	80	32.5	98.8	92.9	74.5
320	8	32	0	80	20.0	100	100	71.4
640	3	37	0	80	7.5	100	100	68.4

Note.TP= True Positive; FN= False Negative; FP= False Positive; TN= True Negative

Table 4 shows that, despite being infected, 27.5% and 35.0% of patients withmelioidosis have negative (below the cut-off value) serological responses for both IgM and IgG respectively at diagnosis ($p< 0.001$).Using the cut-off level of 1:20 for IgM, 72.5% of culture-confirmed patients are seropositive as compared to 10% in Cp and 30% in Ca groups. On the other hand, as for IgG, 65% of culture-confirmed patients were serologically positive as compared to 17.5% in Cp and 7.5% in Ca groups at 1:80 cutoff level. These differences are statistically significant.

Table 4. Seropositivity in Patients with Melioidosis and Control Subjects

Group	M n=40		Control Subject(s)				p-value
			Cp n=40		Ca n=40		
Antibody	Pos. (%)	Neg. (%)	Pos. (%)	Neg. (%)	Pos. (%)	Neg. (%)	
IgM	29 (72.5)	11 (27.5)	4 (10.0)	36 (90.0)	12 (30.0)	28 (70.0)	<0.001
IgG	26 (65.0)	14 (35.0)	7 (17.5)	33 (82.5)	3 (7.5)	37 (92.5)	<0.001

Note. Based on cut-off IFAT-IgM= 1:20, IFAT-IgG= 1:80, M= Patients with melioidosis, Cp= patients with other bacterial infection, and Ca= out-patients without any infection.

Discussion

The cut-off value for IFAT-IgM in the diagnosis of melioidosis, as determined from this study, is determined as 1:20 based on ROC curve. This level is lower than recommended by earlier studies. Lower level of cut off value will increase the rate of detection since the incidence of melioidosis in Malaysia, particularly the Pahang state, is relatively high (4.3 per 100,000) and carries high morbidity and mortality rates (44%).[15] This cut-off value would maximize the sensitivity of the test and at the same time does not encroach upon its specificity, thus it will eventually prevent under-diagnoses of melioidosis.Based on our ROC curves, the cut-off value for IFAT-IgG is determined at higher level which is 1:80 as compared to IFAT-IgM. This is due to the higher background of positive IgG in patients without melioidosis (control group). Our findings confirm another previous local study which showed that IFAT-IgG titers are of most value for prognostic rather than diagnostic purposes.[16]A study done elsewhere also concluded that IFAT-IgMis the most useful marker for diagnosis of an active infection.[17] Although IFAT-IgM is more appropriate than IFAT-IgG for the diagnosis of melioidosis,[18] the IFAT-IgG value is still indispensable when IFAT-IgM is negative.

Furthermore, the area under the ROC curve of about 0.810 for both IFAT-IgM and IFAT-IgG which are more than 0.80 suggests that IFAT method has performed well in discriminating between melioidosis patients and control subjects.[19]The cut-off values for IFAT that had been used in previous studies varied from 16[18], 40[11,17], to 80[19].

All of these values were determined via different methods than ROC which has been extensively utilized in diagnostic tests evaluation as one of the best methods.[20] Therefore, more data is needed to

validate the cut-off value via case control studies.

Based on the selected cut-off values for IFAT-IgM and IFAT-IgG, the majority of control subjects (>70%) have no detectable serological evidence (both IgM and IgG) of melioidosis infection. However, there are 27% to 35% of melioidosis patients have poor or undetectable serological responses which are probably related to their poorly controlled diabetes mellitus status[21] or due to overwhelming sepsis.[22] Thus, IFAT and perhaps other similar serological tests will only be useful as a complementary test. Meanwhile, seropositivity is also observed among control subjects and this is probably due to previous single or repeated exposure to a source of infection[3] caused by *B. pseudomallei*, but without any clinical symptom. Moreover, presence of seropositivity among residents from an endemic area is relatively common and is encountered in any serological assay.[7,22] The IgM and IgG seropositivity detected among control subjects in this study is unlikely to be due to cross-reactivity of IFAT-IgM and IFAT-IgG with other bacterial infections since similar seropositivity is also detected among control subjects without apparent infection.

The two cut-off values determined in this study are more practicable to be applied in endemic areas than in non-endemic or low endemicity areas whereby the specificity shall take priority before selecting the optimal cut-off value. At end of the day, the interpretation of the IFAT results in melioidosis shall be done in the light and context of a wise clinical judgment after detailed history taking and physical examination of a suspected case considering the presence of the risk factors especially diabetes mellitus and exposure to contaminated soil or water. A serological test in general is to be considered only as a guide rather than a stand-alone diagnostic test especially with one such as IFAT that has modest sensitivity and specificity. Nevertheless, the IFAT IgM level is shown to be better than IgG in the diagnosis of melioidosis in the present study.

Conclusion

The in-house IFAT IgM and IgG is a useful method for early serodiagnosis of suspected melioidosis patients at cut-off values of 1:20 and 1:80 respectively. IgM is shown to be of better indicator than IgG and thus recommended.

Conflict of interest: None declared. The authors have no financial, institutional and other relationships that may lead to bias of this article.

Ethical statement: This study was approved by the IIUM Research Ethics Committee (IREC), (IREC 303) and Medical Research Committee (MREC), Ministry of Health (NMRR-14-1141-21985 (IIR)).

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