SERODIAGNOSIS OF MELIOIDOSIS IN SINGAPORE BY THE INDIRECT HAEMAGGLUTINATION TEST

E H Yap, Y C Chan, T Y Ti, T W Thong, A L Tan, M Yeo, L C Ho, M Singh

ABSTRACT

Melioidosis is endemic in Singapore, with diagnosis dependent upon both bacteriological culture and serodiagnosis. Using the polysaccharide (melioidin)-sensitized turkey red cells in the indirect haemagglutination test (IHAT), 20 (100%) of the Pseudomonas pseudomallei culture-positive cases were detectable by the IHAT with titles ranging from 1:16 to 1:32,768. Eight of these patients who died within a few days after the IHAT was performed had titres ranging from 1:16 to 1:1028. Five culture-negative patients, with clinical symptoms suggestive of melioidosis infection and who responded to treatment with ceftazidime, showed IHA titres between 1:64 and 1:8,192. One hundred and twenty one sera from patients with pneumonia, abscesses, or diabetes mellitus were IHAT negative. The IHAT showed good specificity since negative titres were seen in tests using sera from 2 patients with culture-positive Pseudomonas aeruginosa and 4 patients positive for Legionella. IHAT negative results were obtained from tests of 50 normal blood donors and 50 sewerage workers. Of 683 national servicemen tested, 5 (0.73%) had IHAT titres ranging from 1:16 to 1:128. Unlike hyperendemic areas such as Thailand where interpretation of IHAT is seriously hampered by IHA titres found in one-third to half of the population, serodiagnosis of melioidosis by the sensitive IHAT may be employed in Singapore as a routine procedure since backgound IHA titres are low.

Keywords: Melioidosis, indirect haemagglutination test, serodiagnosis of melioidosis by IHAT

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INTRODUCTION

Melioidosis is an infectious disease caused by the Gram-negative bacterium, *Pseudomonas pseudomallei*. Most cases have been reported from Southeast Asia[1-5] and Northern Australia[6,7]. The spectrum of disease ranges from largely asymptomatic, to mild subacute disease with localized lesions to the rapidly septicaemic form. The disease may remain dormant but may recrudesce to the severe fulminant form years after initial exposure[8]. Early diagnosis depends on clinical acumen, isolation and identification of the organism and serodiagnosis. Many serodiagnostic methods have been devised. These include the complement fixation test[9], the indirect haemagglutination test, IHAT[10], IgG- and IgM-IFA[11-13], IgG- and IgM enzyme-linked immunosorbent assay (ELISA)[14-16] and the ELISA test for the exotoxin[17].

Although the IHAT is the simplest and most widely employed, interpretation is hampered by high titres in normal individuals living in hyperendemic areas like Thailand[13]. This study was undertaken to develop a suitable routine technique for the serodiagnosis of melioidosis in a largely urban environment like Singapore.

Microbiology Department
National University of Singapore
Lower Kent Ridge
Singapore 0511

E H Yap, PhD
Associate Professor

Y C Chan, PhD
Associate Professor

T W Thong
Laboratory Technician

L C Ho
Laboratory Technician

M Singh, PhD
Associate Professor

Department of Pharmacology
National University of Singapore

T Y Ti, MBBS, FRCP
Associate Professor

Department of Pathology
Singapore General Hospital
Outram Road
Singapore 0316

A L Tan, MBBS, FRCPA
Registrar

M Yeo, MBBS, DTM&H, DipBact, FRCPA
Consultant

Correspondence to : Assoc Prof E H Yap

MATERIALS AND METHODS

Source of sera. A total of 935 sera were tested by IHAT. Of these, 20 were from acute, septicaemic cases (of whom 8 died within a week after the test was performed) whose blood samples contained *P. pseudomallei*. Five sera were from patients who had pulmonary symptoms suggestive of melioidosis but with negative-blood cultures and responded to treatment with ceftazidime. One hundred and twenty one sera were from patients with pulmonary infections (some with diabetes mellitus) and abscesses. Two sera were from patients from whom *P. aeruginosa* were cultured and 4 from patients tested positive for *Legionella*. Control sera were derived from 683 national servicemen from various camps in Singapore, 50 blood donors and 50 sewerage workers.

Source of antigen. A local isolate of *P. pseudomallei* confirmed by colonial morphology and biochemical reactions using the API 20NE system (API Laboratory Products, U.K.) was used as antigen for the IHAT.

IHA test. The IHA test was a modification of the procedure described by Alexander et al.[10]. The antigen was prepared by the method of Rice et al.[18]. Briefly, *P. pseudomallei* was incubated in a protein-free, chemically defined broth at 37°C for 2w, autoclaved and centrifuged. To the supernatant was added 0.5% phenol and the antigen stored at 4°C. The antigen was coated onto tannic acid-treated, formalinized turkey red blood cells. Optimal dilution of the antigen was determined by checkerboard titration with reference control positive and

negative sera. The antigen was incubated with 1% turkey erythrocytes at 37°C for 30 min and the excess antigen removed by washing twice with phosphate-buffered saline. Sera were inactivated by heating at 56°C for 30 min and absorbed with saline washed nonsensitized turkey erythrocytes at 37°C for 30 min before testing. Doubling dilutions of the serum samples (1:4 - 1:5,096) in 1% heat-inactivated, normal rabbit serum in PBS were made in microtitre plates. To each dilution of serum was added an equal volume of sensitized turkey cells. Results were read within 1 h. Controls, using positive and negative sera, sensitized and nonsensitized cells, were included. The end point was defined as the highest dilution in which haemagglutination occurred.

RESULTS

All patients with melioidosis, confirmed by the isolation and identification of *P. pseudomallei* (from blood or pus) had antibodies detectable by the IHA test (Table I). IHA titres of these patients ranged from 1:16 to 1:32,768. The spread of positive titres was similar in bacteraemic patients who succumbed to fulminant septicaemia as those who recovered. Five patients, who showed clinical symptoms with pulmonary involvement consistent with melioidosis but in whom positive blood cultures were not obtained, had levels of IHA titres ranging from 1:32 to 1:4,096. Two bacteraemic patients who were treated and recovered showed IHA titre of 1:128 in sera tested 6 mo and 12 mo after infection (not in Table).

Table I
Results of IHAT of sera from patients with melioidosis

Disease status (no. tested)	Reciprocal of IHA titre <4	4	8	16	32	64	128	256	512	>512
Clinical melioidosis (25)										
Positive cultures (20)										
*Deceased (8)	-	-	-	1	-	2	2	1	-	2
Live (12)	-	-	-	-	2	3	3	-	2	2
Clinical symptoms only (5)	-	-	-	-	1	-	-	1	-	3

* Patients died within a week after tests were done

Table II
Results of IHAT of sera from patients with pneumonia, abscesses, diabetes, *P. aeruginosa*, *Legionella* and different categories of well persons

Disease status (no. tested)	Reciprocal of IHA titre <4	4	8	16	32	64	128
Patients with pneumonia, abscesses, diabetes mellitus (121)	121	-	-	-	-	-	-
P. aeruginosa (2)	2	-	-	-	-	-	-
Legionella (4)	4	-	-	-	-	-	-
Well persons							
National service-men (683)	678	-	-	2	2	-	1
Blood donors (50)	50	-	-	-	-	-	-
Sewerage workers (50)	50	-	-	-	-	-	-

All sera routinely submitted for IHA test for melioidosis showed negative IHA titres (Table II). These included patients with pneumonia, lung, liver or spleen abscesses and some with diabetes mellitus.

Specificity of the IHA test was evaluated using sera from 2 patients with *P. aeruginosa* and 4 with *Legionella* infections respectively. All showed negative IHA titres.

Of 683 sera from national servicemen tested, 5 (0.73%) were IHA positive, with titres of 1:16, 1:32 and 1:128 (Table II). All sera from 50 blood donors and 50 sewerage workers were negative by the IHA test for *P. pseudomallei*.

DISCUSSION

An increase in incidence of melioidosis has been reported in Singapore[19], affecting mainly older males and a disproportionately higher number of Indians and Malays. Most patients were bacteraemic, with a mortality rate of 72%. Although most of the cases were confirmed by isolation of *P. pseudomallei* from blood, pus or urine, it is desirable to provide a rapid, sensitive and reliable serodiagnostic test for this infection. A variety of serological tests have been described, with the most sensitive being the IgG-ELISA[14]. Owing to the safe, stable and simple preparation of the antigen, and the reported sensitivity and specificity, the indirect haemagglutination test was selected as a routine diagnostic procedure.

The polysaccharide antigen used for the IHA test was found to be remarkably stable, with consistent results obtained even with the antigen stored for three years at 4°C. We elected to use the nucleated turkey erythrocytes since results could be read within 30 min. Heterophile antibodies were detectable in some sera but were readily absorbed out with non-sensitized cells. Complement inactivation was also essential since some sera showed high nonspecific titres if unheated.

In the present study, all (100%) bacteraemic patients were IHA positive, with titres ranging from 1:16 to 1:32,768. There was no obvious difference in range of titres of those who died from those who recovered from the infection. In culturally confirmed cases, 97% of U.S. Army personnel[10], 88.9% of Thais[13] and 92% Australians[14] were reported to have positive IHA titres of up to 1:40,960. In one of our patients who was clinically suspected of having melioidosis, the first blood sample, before the blood culture became positive, showed positive agglutination pattern within 30 min. The agglutination pattern became unstable and appeared to be semi-positive after this time. It was felt that low affinity antibodies were responsible for the unstable pattern of agglutination. Stable agglutination was observed, with no change in titre of serum from the same patient taken a few days after the blood culture became positive for *P. pseudomallei*.

IHA titres appeared to remain high for a long period of time, an observation noted by the above reports. Two of our patients showed high IHA titres when tested 6 mo and 9 mo after initial infection.

Of 126 sera submitted to our laboratory for routine IHA tests, 5 showed positive IHA titres ranging from 1:32 to 1:4,096. These patients had lung infections which responded well to treatment with ceftazidime. Based on data from patients with clinical melioidosis, as low a positive IHA titre of 1:16 may be seen in patients with active infection. The remaining 121 sera gave negative IHA titres. None of these patients developed melioidosis.

The specificity of the IHA test has been previously assessed[10]. Some cross-reactivity, particularly in the IgM-IFA, was observed with serum specimens containing IgM antibodies to *Legionella*[14]. In our study, all 4 sera from *Legionella* patients were found to be negative by the IHAT. The specificity

of the IHAT was also reflected in negative results of two sera from patients with *P. aeruginosa* infection. Cross-reactivity can be expected in tests involving the whole organism, as in the IgM-IFA test, or using crude aqueous extracts of the organism as in the ELISA. The polysaccharide component of *P. pseudomallei* showed exquisite specificity as antigen in the IHAT.

In Thailand, high background IHA titres among blood donors and patients who are not suspected of suffering from melioidosis makes definitive diagnosis difficult, although an IHA titre of 1:1,280 is considered indicative of current melioidosis[13]. In Singapore this does not seem to pose a major problem since all of the blood samples from blood donors and most from national servicemen were IHA negative. Five of 683 (0.73%) national servicemen tested showed IHA titres of 1:16 to 1:128. Of these, only one with a titre of 1:128 was found to have presistent respiratory symptoms and an abscess in the right groin. The remaining four were well and could have been exposed to the organism during training since *P. pseudomallei* have been found in soil samples in Singapore. All 50 sera from old (most of them being in the 50s) sewerage workers were IHA negative. In an earlier study in neighbouring Malaysia, an average of 7.3% (116/1,592) of army recruits, forest aborigines, rubber and oil-palm estate population and groundkeepers were IHA positive with titres of ≥ 1:40. The higher background of IHA titres in Malaysia could be attributed to contact with soil, since the highest prevalence of antibodies was found in persons residing in rice-growing areas.

The excellent sensitivity and specificity of the IHAT in detecting antibodies to *P. pseudomallei* lends itself as a suitable serodiagnostic technique in a largely urban environment like Singapore where about 90% of the population live in high rise apartments. Our findings indicate that only a small percentage of the normal population has been exposed to *P. pseudomallei*. Together with a clinical picture compatible with melioidosis, the IHA test could be employed to detect most cases of melioidosis in Singapore.

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