

BURKHOLDERIA THAILANDENSIS WHOLE CELL ANTIGEN CROSS-REACTS WITH *B. PSEUDOMALLEI* ANTIBODIES FROM PATIENTS WITH MELIOIDOSIS IN AN IMMUNOFLUORESCENT ASSAY

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Abstract. An immunofluorescent assay (IFAT) using whole cell antigen derived from *Burkholderia thailandensis* used for detection of total antibodies to *Burkholderia pseudomallei*, was found to compare favorably with a previous published report on a *B. pseudomallei* IFAT assay. At a 1:20 cut-off titer, the assay had high sensitivity (98.9%) and satisfactory specificity (92.3%), when tested against sera from 94 patients suspected of melioidosis. Sera from 12 patients with culture proven melioidosis gave absolute concordance with the 2 test antigens. No sera from 50 blood donors had a titer of ≥ 20 . Cross-reactivity with patients' sera positive for Chlamydia, Mycoplasma, Legionella and typhoid was not observed, except for 3 sera from typhus patients and one from a patient with leptospirosis. The major advantage of this assay is that the cultivation and preparation of *B. thailandensis* as antigen can be carried out in any laboratory with basic microbiological set-up. The serodiagnosis of melioidosis can be made safe for medical laboratory personnel, particularly in *B. pseudomallei* endemic regions.

Key words: *Burkholderia thailandensis*, *B. pseudomallei*, whole cell antigen, melioidosis, IFAT

INTRODUCTION

Burkholderia pseudomallei is a gram-negative soil saprophyte, which is the etiologic agent of melioidosis, a severe and fatal infectious disease occurring in humans and animals. Melioidosis is difficult to diagnose clinically due to the wide range of associated symptoms and mortality is high among patients with under-

lying risk factors especially diabetes and immunosuppression (Puthuchear and Vadivelu, 2002). Definitive diagnosis of melioidosis has historically been based on the isolation of the bacterium from clinical specimens using culture techniques. Bacterial culture may take up to 4 days and reportedly can have associated problems such as inter-strain and medium dependent variability in colony morphology. While the "gold standard" for diagnosis remains the isolation and identification of the causative bacterium, serological assays have proven useful tools in the presumptive diagnosis of the disease. There are many situations where patients are criti-

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cally ill with fulminating sepsis or when infections are deep seated and no specimens are available, where serology may be sufficiently rapid to facilitate aggressive and appropriate treatment and management of patients.

Serology also has a particular role in subacute presentations and in the screening of patients with febrile illness in endemic areas as well as personnel who have returned from areas of endemicity. Moreover, specific serodiagnosis can also be used to monitor disease activity in patients under treatment by investigating the persistence of antibody levels in serial samples (Vadivelu and Puthuchery, 2000).

We had developed and evaluated an immunofluorescent test (IFAT) for the diagnosis and prognosis of melioidosis using *B. pseudomallei* whole cells as the antigen. The preparation of the whole-cell antigen and test procedures are as described by Vadivelu *et al* (1995). This IFAT test had a sensitivity of 91% and specificity of 100% when compared with culture and performed more efficiently than the indirect hemagglutination test (Vadivelu *et al*, 1995). We have since used this test successfully for the presumptive diagnosis of melioidosis on more than 5,000 serum samples from patients in both East and West Malaysia.

A major disadvantage of the IFAT is the preparation of the *B. pseudomallei* whole cell antigen in that it requires biosafety level 3 (BSL-3) containment facilities for its preparation. Also *B. pseudomallei* has been described as having the potential to be used as a biological weapon. *Burkholderia thailandensis* is genetically and immunologically related to *B. pseudomallei*, but is a relatively avirulent *Burkholderia* species that is non-patho-

genic, or weakly pathogenic in humans (Wiersinga *et al*, 2008) and unlike *B. pseudomallei*, does not require strict biocontainment conditions for study (West *et al*, 2008). Because it may be a useful research surrogate for *B. pseudomallei*, we developed a serodiagnostic test for the detection of antibodies to *B. pseudomallei* in patients with melioidosis, using *B. thailandensis* as antigen, similar to the IFAT mentioned above. The rationale for this was a report that cross-reactivity between sera from culture-positive cases of melioidosis and *B. thailandensis* was demonstrated by the indirect hemagglutination assay using a culture filtrate antigen (Gilmore *et al*, 2007).

This study reports on the use of *B. thailandensis* as antigen in an immunofluorescent assay and compares its sensitivity and specificity to an in-house established IFAT test that uses *B. pseudomallei* whole cells as antigen for the serodiagnosis of melioidosis.

MATERIALS AND METHODS

Bacterial strains and antigen preparation

Four clinical strains of *B. pseudomallei* were pooled together and used for the preparation of the antigen. *B. thailandensis* –ATCC 700388 was used for preparing the second antigen using the same methodology as described by Vadivelu and Puthuchery (1995). Briefly, single colonies of organism were picked into nutrient broth and placed in a shaking incubator at 37°C overnight. The broth culture was then centrifuged and deposited pellets were suspended in 8% formalin for 48 hours to render the organism non-viable. Following washes, bacteria were resuspended in phosphate buffered saline (PBS) and the final antigen concentration was adjusted to 6.4 mg/ml. After a 1,000 fold dilution in PBS, 45 µl of the antigen was

dotted on each well of a Teflon-coated slide and air-dried.

Sera

One hundred sera samples from 94 patients used in this study were collected from the University of Malaya Medical Centre, Kuala Lumpur as well as from other hospitals around Malaysia, and submitted to the Department of Medical Microbiology from 2004-2008, for serodiagnosis of melioidosis. In addition, 12 sera from patients with culture proven diagnosis of melioidosis were also included in the study.

In order to evaluate the specificity of the test using *B. thailandensis* as antigen, known positive disease state serum samples were also assayed. This cross-reactivity panel of 46 sera were obtained from different research laboratories of the Medical Microbiology Department, University of Malaya, from patients who were diagnosed for *Chlamydophila pneumoniae* ($n=7$), *Legionella pneumophila* ($n=6$), *Salmonella typhi* ($n=8$), typhus group rickettsiae ($n=11$), *Mycoplasma pneumoniae* ($n=7$), and *Leptospira* ($n=7$), diseases that are endemic to Southeast Asia. Further 50 sera obtained from healthy blood donors from the Kuala Lumpur metropolitan area, provided by the Blood Bank of the University of Malaya Medical Centre were also included in this study.

Indirect immunofluorescent antibody test (IFAT)

The assay was performed as described by Vadivelu and Puthucherry (2000). Each test serum was serially diluted in PBS from 1:80 to 1:320 for *B. pseudomallei* antigen; and 1:10 to 1:320 for the *B. thailandensis* antigen and allowed to incubate at 37°C for 30 minutes in a moist chamber. Following 3 washes with PBS (pH 7.4), FITC-affinity purified goat, fluorescein-labelled

anti-human immunoglobulin (IgM/IgG) conjugate (Jackson Laboratories, West Grove, PA) was added to the slides, allowed to incubate for another 30 minutes at 37°C, washed 3 times with PBS, then air-dried, mounted with buffered glycerol, and viewed under an ultraviolet microscope at 60x magnification (oil immersion). Positive control sera from *B. pseudomallei* culture-positive patients and negative control sera were included for each test.

Interpretation

Results were scored as 3+, 2+, 1+, or negative compared with positive and negative control sera. The lower limit for a positive cut-off value for *B. pseudomallei* antigen was a score of 1+ at a dilution of 1:80. Sera samples demonstrating a fluorescent intensity of 3+ or 2+ at a dilution of 320 were considered to have a titer of ≥ 320 (Vadivelu and Puthucherry, 2000).

RESULTS

B. thailandensis was used as the antigen in an IFAT test on 100 sera samples that had been received for the serological diagnosis of melioidosis by the established in-house IFAT and 12 known sera from culture positive cases of melioidosis. Table 1 shows the distribution of titer values of both tests, IFAT-*B. pseudomallei* (Bps) and IFAT-*B. thailandensis* (Bts) at various serum dilutions. In the IFAT-Bps, we had used a conservative cut-off value of 1:80 and not 1:40 in order to differentiate true infections from increased background titers due to basal antibody levels in endemic areas. In the present study, 88 patients were seroreactive by the IFAT- Bps at a cut-off titer of 1:80. At this same cut-off titer of 1:80, the IFAT-Bts reacted with only 53 sera samples indicating cross-reactivity between *B. thailandensis* and *B. pseudomallei*. However this was not acceptable as 35 of

Table 1
Results of IFAT using whole cell antigens of *B. pseudomallei* and *B. thailandensis*.

IFAT-Bps titer	IFAT-Bts titer						Total no. serum
	<10	20	40	80	160	320	
<80	12	0	0	0	0	0	12
80	0	10	10	12	3	0	35
160	1	5	9	5	16 [1]*	3	39 [1]*
320	0	0	0	1	1	12 [11]*	14 [11]*
Total	13	15	19	18	20	15	100 [12]*

Bps = *B. pseudomallei*; Bts = *B. thailandensis*

[]* culture positive sera

Table 2
Cross reactivity results.

Cross-reactive panel	Total number	No. (%) IFAT +ve	
		Bps	Bts
Chlamydia	7	0	0
Mycoplasma	7	0	0
Typhoid	8	0	0
Typhus	11	1 (9.0)	3 (27.2)
Legionella	6	1 (16.7)	0
Leptospira	7	1 (14.3)	1 (14.3)
Total	46	3	4

Bps = *B. pseudomallei*; Bts = *B. thailandensis*

the 88 IFAT-Bps positive samples were not reactive. When the cut-off titer for IFAT-Bts was lowered to 1:20, 87 of the 88 samples were considered to be positive and the only one negative IFAT-Bts had a titer of 1:160 for the IFAT-Bps. Thus at a 1:20 cut-off for IFAT-Bts, the sensitivity and specificity were 98.9% and 92.3%, respectively.

Therefore, a titer of ≥ 80 for IFAT-Bps, which is indicative of a presumptive diagnosis for melioidosis, can be assumed to equal an IFAT-Bts titer of ≥ 20 .

Of the 12 sera from culture positive

cases, the IFAT-Bps was 320 for 11 and 160 for one serum. Similar results were obtained with the IFAT-Bts (Table 1).

Of the 50 blood donors, none had an IFAT-Bts titer of ≥ 20 or an IFAT-Bps titer of ≥ 80 . Four sera had IFAT-Bps titers of 1:40 which is considered a negative result and probably due to basal antibody levels in an endemic area.

Cross reactive panel

Twenty-two sera, positive for Chlamydia, Mycoplasma and typhoid, did not show any cross-reactivity with either *B. pseudomallei* or *B. thailandensis* antigen.

Both the IFAT-Bts and IFAT-Bps showed 14.3% (1/7) cross reactivity to *Leptospira* group positive samples, the titers being 1:40 and 1:80, respectively (Table 2) and 16.7% (1/6) *Legionella* positive samples had cross reactivity with IFAT-Bps. The IFAT-Bts showed 27% (3/11) and the IFAT Bps 9% (1/11) cross-reactivity to Typhus positive samples. The titers were 1:40, 1:20 and 1:20 for Bts and 1:160 for Bps.

DISCUSSION

The absence of a well-established universally acceptable antigen has made the serologic diagnosis of melioidosis very difficult and controversial. In a previous study we had reported that an IFAT for the detection of total antibodies to *B. pseudomallei* in patients with melioidosis, using whole cell antigen had a satisfactory sensitivity and specificity when compared with culture (Vadivelu *et al*, 1995). But *B. pseudomallei* has been considered a potential agent for biological warfare and biological terrorism and is listed by the CDC as a Category B bioterrorism agent (Rotz *et al*, 2002). Cultivation and preparation of *B. pseudomallei* as antigen will require biosafety laboratory level 3 (BSL-3) facilities, which are not commonly available in developing countries. Thus a need for the development of a safe diagnostic test was considered. *B. thailandensis* is known to be non-pathogenic and is more closely related to *B. pseudomallei* than to any other *Burkholderia* species. Spectroscopic analysis of purified O polysaccharide from *B. thailandensis* has demonstrated that this organism synthesizes a repeating dissacharide having the structure $\rightarrow 3)-\beta$ -D-glucopyranose-(1 \rightarrow 3)-6-deoxy- α -L-talopyranosyl-(1 \rightarrow 3)-*B. pseudomallei* also produce this repeating dissacharide, as well as a 1,3-linked homopolymer of 2-O-acetylated 6-deoxy- β -D-*manno*-heptopyranosyl

residues. *B. pseudomallei* and *B. thailandensis* are immunogenically and structurally similar and thus give rise to cross-reactive antibody response (Brett *et al*, 1998). *B. thailandensis* LPS was shown to cross-react with rabbit and mouse sera obtained from inoculation with *B. pseudomallei* or *B. mallei*, suggesting that *B. thailandensis* LPS shares similar structural features with LPS molecules from the pathogenic *Burkholderia* species (Qazi *et al*, 2008). Although the immunogenicity of *B. thailandensis* in humans is not known, lipopolysaccharide, the immunodominant antigen of *B. pseudomallei*, is known to be conserved in *B. thailandensis* (Thepthai *et al*, 2001). *B. thailandensis* has not been reported to have been isolated in Malaysia and therefore we will not expect antibodies to this organism to be present in this population.

In the present study, whole cell antigen derived from *B. thailandensis* used in an immunofluorescent assay to detect total antibodies to *B. pseudomallei* was found to have good sensitivity (98.9%) and satisfactory specificity (92.3%). These results compare favorably to the sensitivities and specificities of previous published melioidosis serological assays (Allwood *et al*, 2008). The cross reactivity with Typhus and *Leptospira* does not in any serious way minimize the usefulness of this assay as these diseases can be excluded on a clinical and serological basis. There was a total concordance rate between the 2 tests and isolation of the organism which is the gold standard for diagnosis of melioidosis.

The major advantage of this assay is that the cultivation and preparation of *B. thailandensis* as antigen can be carried out in any laboratory with basic microbiological set-up. Thus, the serodiagnosis of melioidosis can be made safe for medical laboratory personnel, particularly in *B. pseudomallei* endemic regions found mostly

in developing countries. These are preliminary results with relatively small number of samples and larger number of sera from different regions should be tested.

It has been recognised that serodiagnosis is problematic in areas of endemicity due to background seropositivity. We have demonstrated that if a suitable cut-off titer is used to exclude background antibody levels, then a sensitive and specific serological test can lend support to the diagnosis of melioidosis. Of the 88 sero-reactive samples, 23 had titers of 160 or greater, but their clinical status was unknown. This may be an indication of active disease or past exposure where antibody levels remain elevated for prolonged periods after initial infection (Vadivelu and Puthuchear, 2000).

As with all serological assays, the results of IFAT-BTs need to be carefully interpreted and diagnosis should not be determined on the result of serological tests alone, but must also take note of the clinical symptoms, areas of endemicity and other potential factors.

ACKNOWLEDGEMENTS

The authors wish to thank Ms Harvinder Kaur for technical support and assistance. This study was supported by a research grant (FS167/2007C) provided by the University of Malaya, Kuala Lumpur and eSciencefund from the Ministry of Science, Technology and Innovation, Malaysia (no. 12-02-03-2033).

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